Molecular Authentication of Chinese Medicinal Herbs

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Abstract

DNA fingerprints for six *Panax* species, nine *Acorus* species and six *Epimedium* species were generated from random-primed PCRs. Each of these species had characteristic pattern that can be used for identification. Relatedness of the herbs within the same genus was also studied by estimating the genetic distances from the genomic DNA fingerprints. Phylogenetic trees were plotted by UPGMA clustering. *P. quinquefolius* and *P. ginseng* are more closely related than with other species; while *P. trifolius* is distantly related to them. Species of *Acorus* and *Epimedium* can be grouped into clusters.

Attempts had also been made to identify commercial ginseng products by random-primed PCRs. Ten samples were tested. Five of the ginseng products contained only American ginseng and two of them contained Oriental ginseng that were in accordance with the HPLC analysis. The remaining samples produced DNA fingerprints of neither American nor Oriental ginseng.

A rDNA-RFLP method for authentication of *Panax* species was developed. The length of the ITS1-5.8S-ITS2 in *Panax* species is about 640 bp, and the length of ITS1 and ITS2 are 242 bp and 224 bp, respectively. Variations in this region allows the use of restriction enzymes to produce unique RFLP patterns for identification purpose. Two adulterants of ginseng, *M. jalapa* and *P. acinosa* were also sequenced. It is found that the 5.8S subunit is highly conserved between different genera but ITS1 and ITS2 are variable between *Panax* and the two adulterants.

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Abbreviations

AFLP	**amplified fragment length polymorphism**
AP-PCR	**arbitrarily-primed polymerase chain reaction**
ATP	**adenosine 5'-triphosphate**
bp	**base pair (s)**
DAF	**DNA amplified fragments**
CTAB	**cetyltrimethylammonium bromide**
dNTP	**deoxynucleoside 5'-triphosphate**
dATP	**deoxyadenosine 5'-triphosphate**
dCTP	**deoxycytosine 5'-triphosphate**
dGTP	**deoxyguanosine 5'-triphosphate**
dTTP	**deoxythymidine 5'-triphosphate**
DNA	**deoxyribonucleic acids**
EDTA	**ethylenediaminetetraacetic acid**
IPTG	**isopropyl β -D-thiogalactopyranoside**
kb	**kilobase (s)**
PAGE	**polyacrylamide gel electrophoresis**
PCR	**polymerase chain reaction**
PEG	**polyethylene glycol**
RAPD	**random amplified polymorphic DNA**
rDNA	**ribosomal DNA**
RNA	**ribonucleic acid**
RNase	**ribonuclease**
RFLP	**restriction fragment length polymorphism**
S	**Svedberg unit (corresponding to a sedimentation coefficient of 1 to 10^{-13} seconds)**
TEMED	**N,N,N,N-tetramethyl ethylene diamine**
Tris	**Tris [hydroxymethyl] aminomethane**
X-gal	**5-Bromo-4-chloro-3-Indoyl-β-D-Galactoside**

Chapter 1 Authentication of Chinese Medicinal Herbs

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1.1 Introduction

In Hong Kong, 94% of the six million population are Chinese. Among them, over 50% still use Chinese medicine as a complementary or an alternative source of health care (Anonymous, 1991; Wong *et al*, 1993). Since Chinese medicine is not on the list of regulation under the Pharmacy and Poison Ordinance, there is no legal control on the practice of Chinese medicine and the use of medicinal materials. Suppliers or retailers may substitute some expensive herbal medicines with low-cost varieties that may have decrease efficacy or have different pharmacological actions. Some of these adulterants are even poisonous.

In 1989, Hong Kong community was shocked by a herbal poisoning case in which two persons suffered permanent neurological damage after consuming a Chinese decoction supposedly prepared from the root of *Gentiana rigescens* (Long-Dan-Cao) (Fig.1.1).

Fig.1.1. The root of *Gentiana rigescens* (Long-Dan-Cao).

This herb has been used for more than two thousand years to treat ocular irritation, sore throat, etc. without any side-effects if in the right dose. The poisoning case in Hong Kong was due to substitution of *G. rigescens* by *Podophyllum emodi* (Gui-Jiu) (But, 1994) which contains the neurotoxin podophyllotoxin. In early 1996, a person took the root of *P. emodi* (Gui-Jiu) instead of the herb Wei-Ling-Xian (Fig.1.2) from *Clematis* species which is used to treat rheumatism or gout. The misuse of *P. emodi* has also been reported in Kuala Lumper and Taipei. It is reported that the importers in both cities accept a new source of Wei-Ling-Xian without realizing the toxicity of the adulterant.

Another case of renal toxicity was due to the substitution of a herb Fang-Ji, the root of *Stephania tetrandra* by Guang-Fang-Ji derived from the root of *Aristolochia* fangchi which contains aristolochi acid. It is known to produce nephrotoxic reactions (De Smet, 1992).

Fig.1.2. The herb Wei-Ling-Xian of *Clematis* species.

In order to prevent the occurrence of similar poisoning cases, measures should be taken at the legal, community and scientific levels to safeguard the healthy development of herbal industry. In 1989, a Working Party on Chinese Medicine appointed by the Secretary of Health and Welfare was set up to monitor the practice of traditional Chinese medicine in Hong Kong. The Party aims at the health education in the proper handling, processing and use of Chinese medicines, the drawing up of a 'potent herbs' list to facilitate control, and the introduction of regulation and registration for practitioners of Chinese medicine (Anonymous, 1991). A list of potent herbs was published (Appendix I). Only qualified practitioners and dispensers are allowed to prescribe and handle these herbs. Apart from the herbal safety surveillance, a well developed quality control system is urgently needed to ensure the authenticity and quality of Chinese medicines.

Traditional identification of Chinese medicinal herbs mainly depends on morphological, anatomical or histological characters. However, they could hardly satisfy the need of the herbal industry as many medicinal plants are processed into powder or shredded pieces. With the advancement of analytical techniques, chemical characterization of active ingredients using Thin Layer Chromatography (TLC) and High Pressure Liquid Chromatography (HPLC) have become routine procedures. Recently, various molecular methods such as polymerase chain reactions, restriction fragment length polymorphisms play a role in genetic detection in rice (Wang *et al*,1994), classification of crop species such as celery (Yang & Quiros, 1993) as well as phylogenetic studies of aphid (Puterka *et al*, 1993).

1.2 Traditonal Identification of Chinese Herbs

1.2.1 Morphology

Traditional means of identification includes the inspection of morphological markers such as shape, colour, texture and odor of the herbs. Although this approach is

the simplest and the most direct, the accuracy depends heavily on the individual's experience. The assessment markers can be subtle and ambiguous. Moreover, the samples are affected by the geographical environment and the period of growth.

1.2.2 Histology

The use of histological techniques provides an alternative means but its application is limited to herbs that are not damaged during harvesting and processing. Structural characterization of organs such as root, leaf as well as the arrangement of cytoplasmic materials in cells enables the identification of plants. The use of scanning and transmission electron microscopes explores ultrastructures such as pollen grain as a potentially useful identification marker.

1.2.3 Chemical Analysis

A more objective authentication approach is the use of chemical analysis such as TLC and HPLC. Both methods assess the profile of characteristic component(s) present in the plants. These methods have been used to identify many medicinal plants including ginsengs (Soldati & Sticher, 1980) (Figs. 1.3 & 1.4). They have also been successfully applied to identify herbal remains in the herbal poisoning cases mentioned above. However, the quantity of the active components is affected by the physiological conditions and the age of the herb as well as the harvesting period and the storage conditions. Sometimes, the profile is too complicated to analyse and difficult to reproduce. Moreover, closely related herbs containing similar chemical components may confuse the identification.

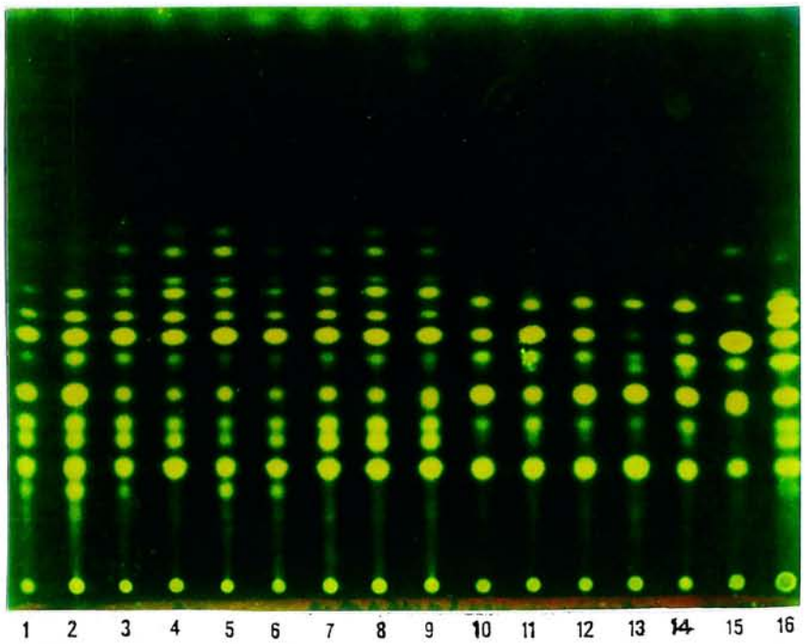

Fig.1.3. A profile of the active components, ginsenosides, in three *Panax* species generated by Thin Layer Chromatography. Lanes 1-9: *P. quinquefolius*. Lanes 10-14: *P. ginseng* and Lane 15: *P. notoginseng*. Lane 16: ginsenosides standard (Xie, 1993).

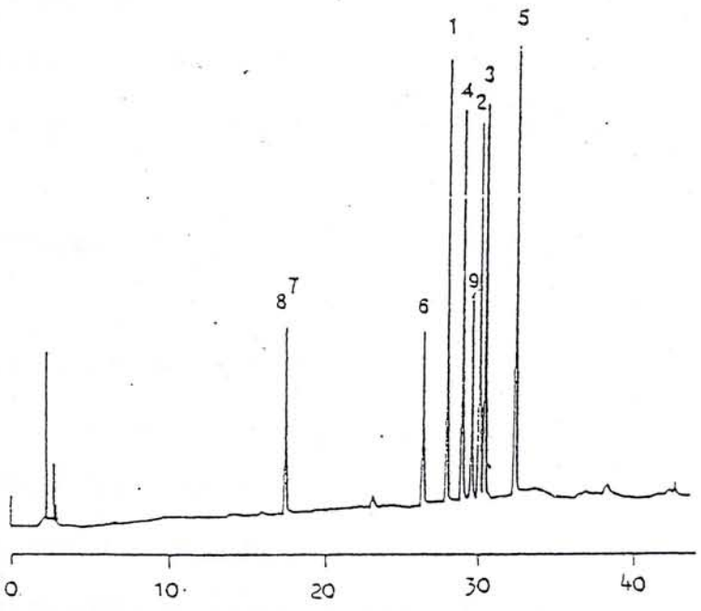

Fig.1.4. A chemical profile of standard ginsenosides of *Panax* species generated by High Pressure Liquid Chromatography (Lang *et al*, 1993).

1.2.4 Proteins and Isozymes

Soluble proteins and isozymes have been reported in authentication of many plants including celery (Quiros *et al,* 1987a, b), cereals (Ragot & Hoisington, 1993), garlic (Maaß & Klaas, 1995) and tree (Wang *et al*, 1996).

By gel electrophoresis, different isozyme profiles can be generated from different herbal samples. Koga (1991) has successfully distinguished *Epimedium diphyllum* from other species by comparing the isozymes of isocitrate dehydrogenase, glutamate dehydrogenase, phosphoglucose isomerase and phosphoglucomutase. However, the informative markers for closely related cultivars are limited. In addition, protein patterns may vary in different tissues, developmental stages and environment as a result of temporal and spatial gene expression.

1.3 Molecular Technology in Authentication

The advancement in molecular biology offers an additional tool in authentication of Chinese herbs. Restriction Fragment Length Polymorphism (RFLP) and Polymerase Chain Reaction (PCR) related techniques are the pioneer methods.

1.3.1 Restriction Fragment Length Polymorphism (RFLP)

RFLP has been applied to identify genetic markers in plants (Edwards *et al*, 1992; Tanksley *et al* ,1989) such as rice (Wang *et al*, 1992; Zhang *et al*, 1992), barley (Zhang *et al,* 1993) and sea beet (Raybould, 1996). It is based on the loss or gain of a restriction site owing to mutation within the enzyme recognition sequence. Sample DNA is digested with various restriction enzymes to produce banding patterns for differentiation (Fig. 1.5). It gives a much higher degree of polymorphisms and consistence. However, the procedures of RFLP are laborious, the cost is relatively high and may involve the use of radioisotopes.

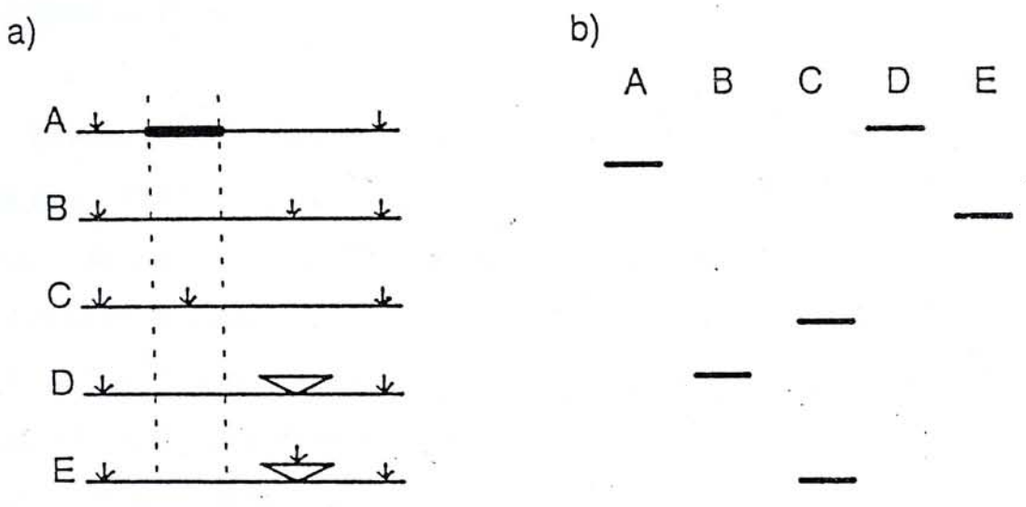

Fig.1.5. Diagrammatic representation of restriction fragment length polymorphisms (RFLP). Part (a) shows the distribution of restriction sites (arrowed) in a piece of DNA in five taxa lines(A to E), containing a region homologous to a cloned probe (shaded). B and C have restriction sites not present in A. D and E have insertions. Part (b) shows the banding pattern from Southern blots of the five taxa after their genomes have been restricted at the sites shown in (a) and hybridized with a probe homologous to the shaded region.

1.3.2 Polymerase Chain Reactions (PCRs)

The use of PCR to amplify DNA has become more and more popular. It is an enzymatic method of multiplying a specific segment of DNA. The amplification is achieved with two synthetic oligonucleotide primers that flank the sequence of target region in DNA. A single PCR cycle involves denaturation of the template DNA, followed by annealing of the primers to their targets on the single-stranded templates. The annealed primers are then extended on the template strands by thermostable DNA polymerase. Repeated cycles of heating and cooling lead to a chain reaction. An exponential accumulation of the DNA fragments would form a discrete band after electrophoresis (Fig.1.6). This technology uses a minute amount of DNA, usually in the order of nanogram. It is particularly relevant to Chinese medicinal plants as they are always dried and processed. The DNA may somehow be degraded.

Several PCR-derived methods for identification of species have been reported.

1.3.2.1 Random-Primed PCRs

Essentially there are three such methods, namely RAPD: Random Amplified Polymorphic DNA (Williams, 1990), AP-PCR: Arbitarily-Primed Polymerase Chain Reactions (Welsh, 1990), and DAF: DNA Amplification Fingerprinting (Caetano-Anolles *et al*, 1991a,b). A single primer, generally 5 to 8 bases in length in DAF, 9 to 10 bases in RAPD and 20-30 bases long in AP-PCR, with an arbitrary sequence is used to amplify a template DNA. Amplification with this single primer under reduced stringent conditions can give rise to a finite amount of DNA fragments which can be identified by gel electrophoresis. The origin of these fragments is unknown but they presumably contain DNA sequences complementary to the 3'end of the primer sequence. Variations in the degree of the homology, the length between the primer sites and changes in the secondary structure between or flanking the primers recognition sites generate molecular polymorphisms for different target DNA.

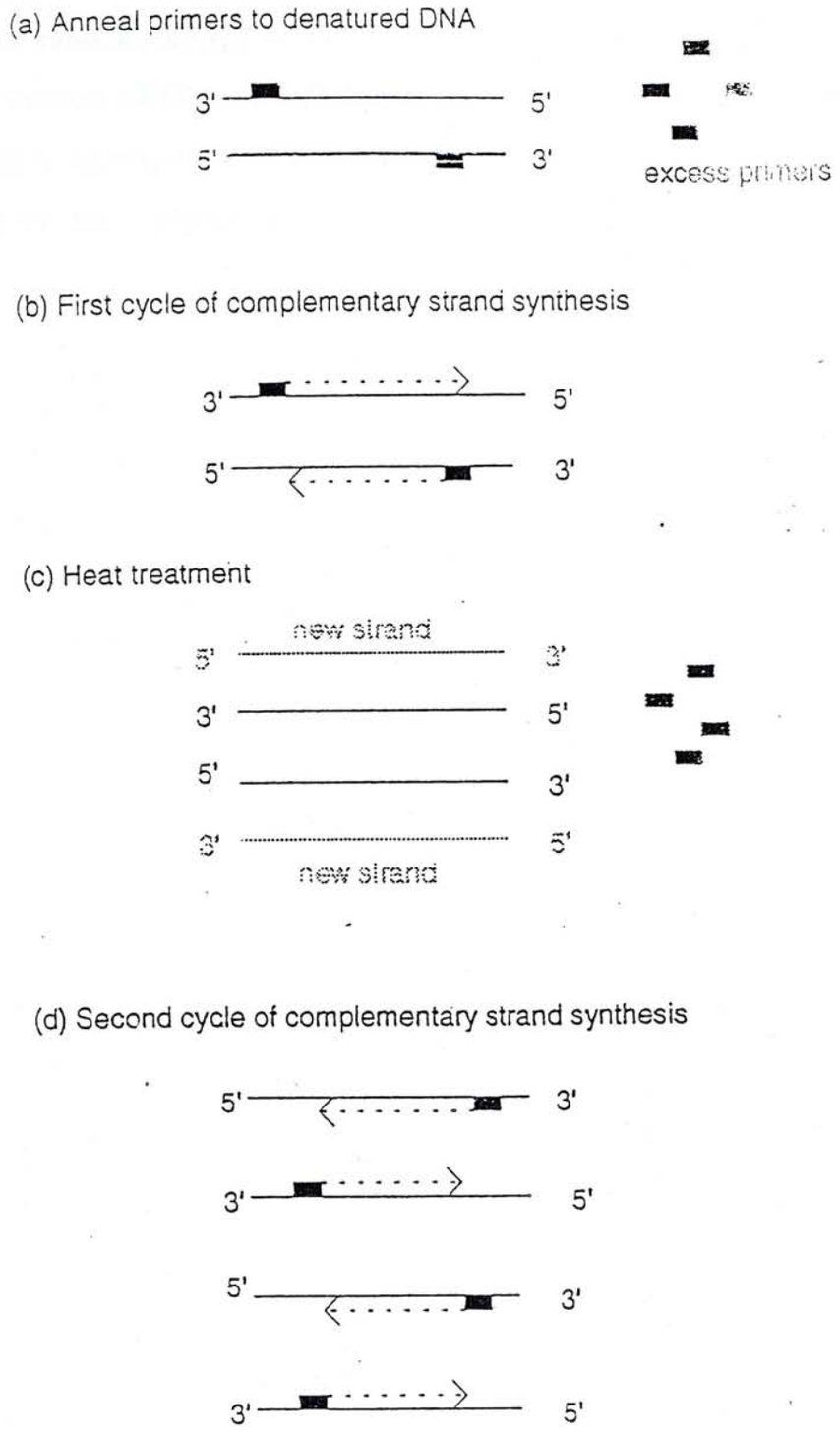

Fig.1.6. Schematic representation of a polymerase chain reaction. (a) A double-stranded DNA molecule is denatured under a high temperature; then two oligonucleotide primers (short black boxes) bind to specific sequences flanking the segment to be amplified. (b) The primers are then extended by DNA synthesis (dashed lines) with *Taq* polymerase. (c) The reaction mixture is heated to 95^0C so that the newly synthesized strands detach from the templates. (d) On cooling, more primers anneal at their respective positions and the cycle is repeated. The reaction can be continued through 30-40 cycles, with DNA amplification proceeding in an exponential fashion.

These methods overcome problems associated with RFLP and conventional PCR. By using the random-primed PCRs, no prior knowledge of the sample DNA is required and minute amount of DNA is sufficient to produce polymorphic patterns. They provide a quick and easy approach to explore genetic polymorphisms. Sometimes, two primers are used. This pairwise combination of primers may enable the detection of more polymorphic markers.

Random-primed PCRs have been widely used in genotyping of bacteria (Wang *et al*,1993), fungi (Tigano-milani *et al*, 1995), crops (Hu and Quiros, 1991a), and mouse (Welsh *et al*, 1991). Our groups have used these methods in differentiating various Chinese herbs such as *Panax* species (Cheung *et al*, 1994; Shaw & But, 1995), *Elephantopi* species, *Taraxacum* species (Cao *et al*, 1996a, b) and *Astragalus* species.

1.3.2.2 Simple Sequence Repeats

Microsatellites refer to tandem repeats on a basic motif of less than six base pairs; for examples, (AC)n, (TCT)n, (TCTG)n. It has emerged as an important source of genetic markers for eukaryotic genomes (Condit & Hubbell, 1991; Rothuizen *et al*, 1994). In plants, microsatellites have been documented in barley (Saghai-Maroof *et al*, 1994), wheat (Röder *et al*, 1995) and grape (Bowers *et al*, 1996). They can be obtained by searching databases such as GenBank (Weber, 1990) and EMBL (Lagercrantz *et al*, 1993) or by screening genomic libraries with synthesized oligonucleotides (Cornell *et al*, 1991). It can also be achieved by screening a library enriched with repetitive sequences isolated from genomic DNA. Repetitive sequences are enriched from total genomic DNA by their annealing temperature characteristics which is termed low-C_0t DNA (Leung *et al*, 1994). Based on the unique sequence flanking microsatellites, primers are designed to detect the microsatellite loci through PCR with genomic DNA or used as a probe in DNA fingerprinting. Röder (1995) has reported a high level of information for polymorphic microsatellite markers in wheat.

1.3.2.3 Amplified Fragment Length Polymorphism (AFLP)

In 1993, Zabeau and Vox developed a novel PCR-based fingerprinting technique called AFLP. This technology is based on the selective amplification of a subset of genomic restriction fragments by PCR. Genomic DNA is digested with restriction endonuclease, ligated to adapters and amplified by PCR using primers that anneal to adapter sequences with additional selective nucleotides on the 3'end of the primers (Fig 1.7). The amplified DNA fragments are separated by denaturing polyacrylamide gel electrophoresis to reveal polymorphisms. The number of resulting amplified DNA fragments is determined by the number and composition of selective nucleotides, as well as the complexity of the genomic DNA. AFLP has been evident as a source of potential molecular markers in fungi (Mueller *et al*, 1996), plant and bacteria (Lin & Kuo, 1996).

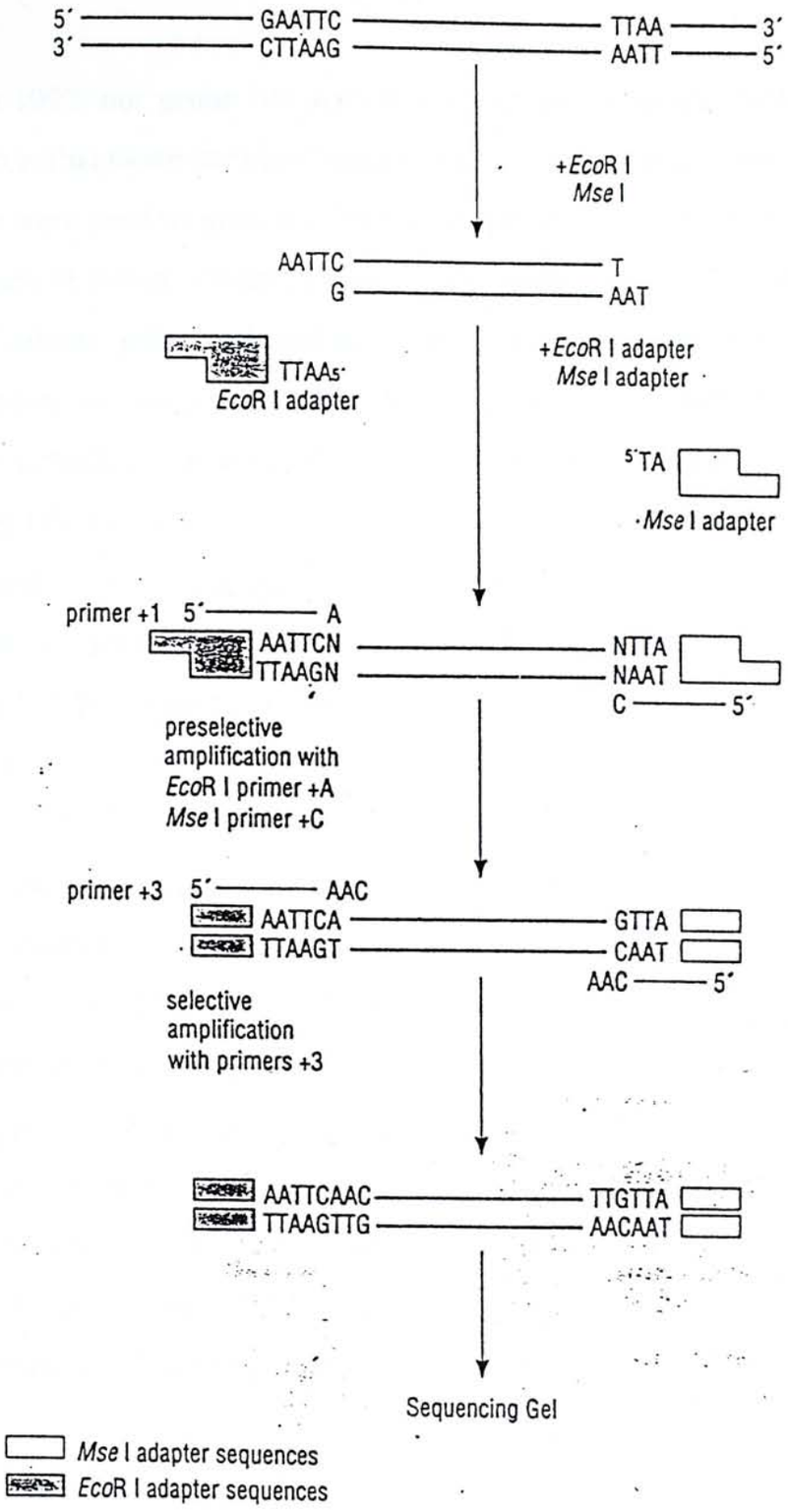

Fig.1.7: A diagram showing the AFLP procedure using one primer (Lin & Kuo, 1996).

1.4 Objectives and Strategies of the Study

Since 1992, our group has started to adopt the molecular techniques of AP-PCR and RAPD to authenticate various Chinese herbs and their adulterants. In this thesis, these two methods were used to generate DNA fingerprints for *Acorus* and *Epimedium* as well as more species of *Panax*. On the market, both American and Oriental ginsengs are often packaged as tablets, pills or shredded pieces. We have also explored the possibility of using the above techniques to authenticate commercial materials. A more accurate approach for authentication has also been developed. The intergenic transcribed region of the ribosomal DNA of various *Panax* species and their adulterants were amplified with primers primed to the conserved 18S and 26S of rDNA. They were sequenced and variation in the sequences were detected. Specific enzymes were then chosen to digest the amplified DNA. The fingerprints generated by PCR-RFLP were also used as a means for authentication.

With the DNA fingerprints and sequences available, we can also investigate the phylogenetic relationship among the plants studied. There are numerous methods for phylogeny inference. They can be divided into two categories: distance and discrete-character methods. In distance methods, a pairwise evolutionary distance is computed for all pairs of operational taxonomic units, which can be either species or populations, or DNA/amino acid sequences. In discrete-character methods, data with discrete characters such as the presence or absence of bands in fingerprints are used. A phylogenetic tree is constructed by algorithm methods such as Unweighted Pair Group Method using Arithmetic means (UPGMA) or Neighbor-Joining (NJ).

UPGMA form groups by successively pairing similar molecular phenotypes according to the magnitude of their observed distances (Sneath & Sokal, 1973), and it computes the standard errors of interior branch length under the assumption of constant evolution rate. The tree is first constructed by linking the least distant pairs of taxa, followed by successively more distant taxa or groups of taxa. When the two taxa are

linked, they lose their individual identities and are subsequently referred to as a single cluster. Initially, each taxon constitutes its own cluster. At each stage in the process, as two clusters are merged into one, the number of clusters declines by one. The process is complete when the last two clusters are merged into a single cluster containing all of the original taxa.

In NJ-tree, the branch lengths of the tree are re-estimated by using the ordinary least squares method and the standard errors of the estimates are computed (Saitou & Nei, 1987). The tree is constructed by linking the least distant pair of nodes as defined by the re-estimated matrix. When two nodes are linked, their common ancestral node is added to the tree and the terminal nodes with their respective branches are removed from the tree. This pruning process converts the newly added common ancestor into a terminal nodes on a tree of reduced size. At each stage in the process, two terminal nodes are replaced by one new node (corresponding to an internal node on the final tree). The process is complete when two nodes remain, separated by a single branch.

Chapter 2 Materials and Methods

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2.1 Reagents and Buffers

2.1.1 Buffers for Total DNA Extraction

- **CTAB Extraction Buffer**

  50mM Tris-HCl (pH 8.0)

  0.7M NaCl

  10mM EDTA

  1% (w/v) CTAB

  20mM 2-mercaptoethanol

- **CTAB Precipitation Buffer**

  50mM Tris-HCl (pH 8.0)

  10mM EDTA

  1% (w/v) CTAB

- **10% CTAB**

  10% (w/v) CTAB

  0.7M NaCl

- **Extraction Buffer**

  100mM Tris-HCl (pH 8.0)

  100mM EDTA (pH 8.0)

  250mM NaCl

- **10% Sarkosyl (N-Lauroyl-sarcosine)**

  10%(w//v) sarkosyl in water

- **TE Buffer**

  10mM Tris-HCl (pH 8.0)

  1mM EDTA

2.1.2 Reagents for Agarose Gel Electrophoresis.

- **TAE (Tris-acetate) 1X**

  40mM Tris-acetate

  1mM Na_2EDTA

- **TBE (Tris-borate) 1X**

  90mM Tris-borate

  2mM Na_2EDTA

- **6X Agarose gel loading Buffer**

  40% (w/v) Surcose

  0.25% (w/v) Bromophenol blue

2.1.3 Reagents for Polyacrylamide Gel Electrophoresis

- **40% Polyacrylamide Solution**

  38% (w/v) Acrylamide

  2% (w/v) N,N'-methylene-bisacrylamide

- **10% Ammonium Persulfate**

  10% (w/v) Ammonium persulfate in distilled water

2.1.4 Reagents for Plasmid and Single-Stranded DNA Preparation

- **P1**

  50 mM Tris-HCl (pH 7.5)

  10 mM Na_2EDTA

  100ug/ml RNaseA

- **P2**

  0.2M NaOH

  1% (w/v) SDS

- **P3**

  2.55M KAc

  adjust to pH 4.8 with glacial acetic acid

- **QBT (pH 7.0)**

  750mM NaCl

  50mM MOPS

  15% Ethanol

  0.15% (v/v)Triton X-100

- **QC (pH 7.0)**

  1M NaCl

  50mM MOPS

  15% (v/v) Ethanol

- **QF**

  1.2M NaCl

  50mM MOPS

  15% (v/v) Ethanol

- **20% PEG**

  20% (w/v) PEG 6000

  0.7M NaCl

  Sterilized by filtration

- **3M Ammonium Acetate**

  3M ammonium acetate

  adjust to pH5.5 with glacial acetic acid

2.1.5 Media for Bacterial Culture

- **Luria-Bertani (LB) Medium**

  10 g/l Tryptone

  5 g/l Yeast extract

  10 g/l NaCl

  Sterilized by autoclave at 121^{0}C for 15 minutes

- **Luria-Bertani (LB) Agar**

  10 g/l Tryptone

  5 g/l Yeast extract

  10 g/l NaCl

  1.5% (w/v) Lacto agar

  Sterilized by autoclave at 121^{0}C for 15 minutes

- **Psi Medium**

  2% (w/v) Tryptone

  0.5% (w/v) Yeast extract

  20mM $MgSO_4$

  10mM NaCl

  5mM KCl

  Sterilized by autoclave at 121^{0}C for 15 minutes

- **2TY Medium**

  1.6% (w/v) Tryptone

  1% (w/v) Yeast extract

  85mM NaCl

  Sterilized by autoclave at 121^{0}C for 15 minutes

- **H-top Agar**

  1% (w/v) Tryptone

  137mM NaCl

  0.7% (w/v) Lacto-agar

  Sterilized by autoclave at 121^0C for 15 minutes

2.1.6 Reagents for Preparation of Competent Cells

- **RF1**

  100mM RbCl

  50mM $MnCl_2.4H_2O$

  30mM KAc

  10mM $CaCl_2.2H_2O$

  15% (v/v) Glycerol

  Adjust to pH 5.8 with glacial acetic acid, sterilized by filtration

- **RF2**

  10mM MOPS

  10mM RbCl

  75mM $CaCl_2.2H_2O$

  15% (v/v) Glycerol

  Adjust to pH 6.8 with NaOH, sterilized by filtration

2.1 DNA Isolation

2.1.1 Sample Preparation. The dried samples were rinsed with 70% ethanol and then distilled water to remove any contaminants. The samples were then ground into fine powder in liquid nitrogen in a mortar and pestle.

2.1.2 Cetyl triethylammonium bromide (CTAB) Extraction. Plant DNA was extracted by a protocol modified from Draper and Scott (1988). 0.1 to 0.2 g ground sample powder was added into 600 µl 1x CTAB extraction buffer prewarmed to 56^{0}C for 30 minutes with occasional shaking, it was then cooled to room temperature and extracted with an equal volume of chloroform/isoamyl alcohol (24:1). After spinning at 13,000 g for 10 minutes, 0.1 volume of 10% CTAB was added into the aqueous phase. It was then extracted again with an equal volume of chloroform/isoamyl alcohol (24:1). The aqueous phase was collected and an equal volume of 1x CTAB precipitation buffer was added. After standing at room temperature for 1 hour, the solution was spun down at 13,000 g for 15 minutes. The pellet was resuspended in 400 µl 1M NaCl, 800µl. of cool absolute ethanol was added and stored at -20^{0}C overnight. The suspension was spun down at 13,000 g for 10 minutes and the pellet was washed with 70 % ethanol twice. It was then air-dried and resuspended in 50 µl TE buffer or double distilled water.

2.1.3 Cesium Chloride Gradient Ultracentrifugation.

2.1.3.1 Cell Lysis. 1 to 2 g ground sample powder was added into 10 ml extraction buffer. 1 ml 10% Sarkosyl and 100 µl (10 mg/ml) proteinase K were added. The mixture was incubated at 56^{0}C for 1 to 2 hours. It was then centrifuged in a Beckman JA-21 at 13,000 g for 20 minutes to remove cell debris.

2.1.3.2 Precipitation of DNA. 0.6 volume of isopropanol was added to the supernatant and gently mixed. A nucleic acid precipitate should be visible; otherwise it was placed at -20^{0}C overnight for convenience. It was centrifuged at 13,000 g for 20 minutes. The supernatant was drained out and the pellet was resuspended in 4 ml TE buffer.

2.1.3.3. Purification of DNA. 4.9 g solid cesium chloride was added and mixed gently until it was dissolved. The lysate was incubated on ice for 30 minutes and 0.25 ml of 10 mg/ml ethidium bromide was added and incubated on ice for 30 minutes. It was then centrifuged at 13,000 g for 15 minutes at 4^{0}C. The supernatant was transferred into a 5-ml ultracentrifugation tubes. It was centrifuged in a Beckman NVTi 65.2 rotor at 350,000 g overnight at 20^{0}C.

2.1.3.4 Collection of DNA. DNA was collected using a 18G needle and syringe. The cap of the ultracentrifuge tube was removed and the needle was inserted just below the DNA band, which was then removed. The ethidium bromide was removed by repeatedly extracting the collected DNA with water-saturated butanol until the solution became colourless. The salt in the DNA solution was removed by dialysis with 2 changes of TE buffer overnight.

2.3 Phenol/Chloroform Extraction

Equal volume of phenol:cholorform: isoamyl alcohol (25:24:1) was added to the suspension to denature protein. It was gently shaken to mix the two phases. The phases were then separated by centrifugation at 13,000 g in a bench-top centrifuge for 15 minutes at room temperature. The aqueous phase was withdrawn , leaving behind any precipitate at the interface. It was then extracted with equal volume of chloroform by inversion and centrifuged at 13,000 g for 10 minutes. The aqueous phase was then subject to ethanol precipitation.

2.4 Ethanol Precipitation

DNA solution was precipitated with 0.1 volume of 3M sodium acetate and 2.5 volumes of cold absolute ethanol at -20^{0}C overnight. It was pelleted by centrifugation at 13,000 g at 4^{0}C for 10 minutes. The pellet was washed with 70% (v/v) ethanol and air-dried. It was then resuspended in an appropiate volume of TE buffer or double-distilled water and stored at -20^{0}C.

2.5 DNA Concentration/Purity Estimation

The size of DNA was checked by running 5 µl sample onto 1% agarose horizontal gel for 1 hour at 10V/cm in TBE buffer. Lambda *Hin*d III digested DNA was used as a size marker. The intensity of the band indicated the relative quantity of DNA extracted. Any smear present in the lane showed the quality and purity of the DNA. The concentration and purity were also checked using UV spectrophotometer at wavelength 260 nm and 280 nm. The amount of UV absorbed by a DNA solution is directly proportional to the amount of DNA in the sample. Its concentration can be determined by the formula:

$$[DNA] = A_{260} \times \text{dilution factor} \times 50\ \mu g/ml$$

One A_{260} unit corresponds to 50 µg of double-stranded DNA per ml. The A_{260}/A_{280} ratio of 1.8 indicates a reasonably pure DNA. Ratios less than 1.8 indicate that the preparation is contaminated either with protein or phenol.

2.6 Random-Primed Polymerase Chain Reactions

2.6.1 Random Amplified Polymorphic DNA (RAPD). It was performed in 25 µl of 10-25 ng plant DNA, 0.1 mM dNTPs, 0.2 µM primer, 1x *Taq* buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.001% gelatin), and 0.5 units of *Taq* polymerase. Reaction was taken place in a Thermolyne thermocycler through 45 cycles of 94^0C, 1 min; 36^0C, 2 min; 72 ^{0}C, 2 min.

2.6.2 Arbitarily-Primed Polymerase Chain Reaction (AP-PCR). It was performed in 25 or 50 µl of 10 to 100 ng plant DNA, 0.2 mM dNTPs, 2 µM primer, 1X *Taq* buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.001% gelatin), and 1 unit *Taq* polymerase for 25 µl or 2.5 units for 100 µl. Reaction was taken place in a Thermolyne thermocycler or MJ-PTC 100 through the following cycles: Cycles 1 and 2: 94^0C, 5 min; 40^0C, 5 min.; 72^0C, 5 min. Cycles 3 to 42: 94^0C, 1 min; 60^0C, 1 min; 72^0C, 2 min. Cycles 43: 94^0C, 0.5 min.; 60^0C, 0.5 min., 72^0C, 10 min.

After the PCRs, the products were resolved onto a 2.5% TBE agarose gel and photographed by polaroid 665 films.

2.7 rDNA Amplification

The rDNA was amplified using a pair of primers 18d and 28cc on the DNA encoding the 18S and 26S subunits respectively.

18d: CACAC CGCCC GTCGC TCCTA CCGA

28cc: ACTCG CCGTT ACTAG GGGAA

The reaction was performed in a 50 µl mixture containing 1 ng plant DNA, 1x *Taq* buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.001% gelatin), 0.2 mM dNTPs, 1 µM of each primer and 1 unit of *Taq* polymerase. Reaction mixtures were overlaid with mineral oil and reaction was carried out in a Thermolyne thermocycler. Initial template denaturation was programmed at 94^{0}C, 5 min. It was then subject to 35 cycles of 94^{0}C, 1 min; 60^{0}C, 1 min; 72^{0}C, 2 min and with a final extension of 72^{0}C, 10 min.

After the reaction, the products were resolved by a 1.4% TBE agarose gel.

2.8. Agarose Gel Electrophoresis of DNA

DNA was separated by 1 to 2.5% TBE /TAE agarose gel, which was prepared by dissolving 1 to 2.5% agarose (w/v) in 1X TBE or TAE containing 0.5 µg ethidium bromide/ml. The DNA sample was mixed with appropriate amount of 6X agarose loading buffer to give a final concentraion of 1X. Then the sample was loaded into the slots of the gel which was placed into the electrophoresis tank with 1X TBE or 1X TAE buffer. It was run at 5-10 V/cm. After the bromophenol blue was migrated to appropriate distance, the gel was examined under ultraviolet light. It was photographed by polariod 665 films.

2.9 Purification of rDNA

2.9.1 from Agarose Gel using Geneclean® II Kit (Bio 101 Inc.). The procedure was recommended by the supplier. The desired DNA band separated by TAE gel was cut out into pieces using a razor blade. The weight of the gel slices was determined and 3 volumes of 6M sodium iodide solution was added. The gel slices were melted at 55^{0}C for about 5 minutes and 5-10 µl glassmilk was added. The mixture was kept on ice for 15 minutes. It was centrifuged in a microcentrifuge at 12,000 g for 30 seconds. The pellet

was washed with 0.7 ml New Wash solution. The suspension was pelleted by centrifugation. The pellet was washed two more times and then air-dried to remove the traces of ethanol. Then 20-50 µl double-distilled water prewarmed at 56^{0}C was added to elute the DNA. It was collected by centrifugation at 12,000 g for 1 minute.

2.9.2 using Microspin^{TM} Columns. The procedure was recommended by the supplier. The resin in the column was resuspended by vortexing. It was placed in a microfuge and prespun at 735 g in a microcentrifuge for 1 minute. The microfuge was replaced with a new one and the sample was applied slowly to the top-center of the resin. It was spun at 735 g for 2 minutes. The purified sample was collected at the bottom of the support tube.

2.10 Preparation of *Escherichia coli* Competent Cells

Appropriate strain of *E.coli* was streaked directly from frozen stock onto a LB agar plate and incubated at 37^{0}C overnight. A single colony from the plate was inoculated into 10 ml Psi medium and shaken vigorously at 37^{0}C for 3 hours. Then 10 ml culture was added into 90 ml Psi medium. The cells were allowed to grow at 37^{0}C until the O.D. reached 0.5. The culture was chilled on ice for 15 minutes and then harvested by centrifugation at 10,000 g for 15 minutes at 4^{0}C in a Beckman superspeed centrifuge J2-21, rotor JA20. The pellet was resuspended in 8 ml RF 2 and kept on ice for 15 minutes. The competent cell suspension was dispensed into aliquots of 100 µl each and frozen in liquid nitrogen immediately. They were then stored at -70^{0}C before use.

2.11 Ligation and Transformation of *Escherichia coli*

The purified PCR product was ligated into 25-100 ng vector in a ratio of 3:1. The reaction was incubated at 16^{0}C overnight. 5 µl of the ligation mixture was added into 100 µl of thawed *E.coli* competent cells and mixed by stirring with a pipette tip. The tube was kept on ice for 30 minutes and then heat-shocked at 42^{0}C for 2 minutes. It was rapidly transferred to ice to chill the cells for 2 minutes. Then 400 µl of pre-warmed at 37^{0}C LB medium was added to the tube and incubated at 37^{0}C for 1 hour in a rotary shaking incubator. 50 µl of the culture, 50 µl of 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and 20 µl of 0.1M isopropylthio-β-D-galatoside (IPTG) were spreaded onto a LB plate containing appropiate antibiotics. After the liquid had been diffused into the agar, it was inverted and incubated at 37^{0}C overnight.

2.12 Isolation of Plasmid DNA

A single bacterial colony was inoculated into 5 ml LB medium containing appropiate antibiotics. The culture was incubated at 37^{0}C overnight in a rotary shaking incubator. 1 to 3 ml bacterial cells were collected by centrifugation at 13,000 g for 30 seconds. The pellet was resuspended in 200 µl of Buffer P1 containing 100 µg/ml RNaseA. 200 µl of Buffer P2 was added to lyse the cells which was then allowed to stand at room temperature for 5 minutes. 200 µl of Buffer P3 was added to neutralize the mixture and kept on ice for 5 minutes. It was centrifuged at 13,000 g at room temperature for 15 minutes. The supernatant was collected and 0.7 volume of isopropanol was added to precipitate the DNA at room temperature. After 30 minutes, it was centrifuged at 13,000 g for 15 minutes. The pellet was washed with 70% ethanol twice and then air-dried. It was then dissolved in 20-50 µl double distilled water. (The constituents of buffers P1, P2 and P3 are mentioned in Section 2.1.4).

2.13 Screening of Plasmid DNA by Restriction Digestion

2 µl of plasmid was digested with 0.2 unit enzyme in 20 µl containing appropiate buffer at 37^{0}C for 1 hour. It was resolved in a 1.4% TBE agarose gel.

2.14 Isolation of Plasmid DNA

2.14.1 Minipreparation of Plasmid using Magic ^{TM} Miniprep DNA Purification Kit (Promega). The following procedure was recommended by the supplier. A single colony was inoculated in 3 ml LB medium containing the appropriate antibiotic. It was incubated at 37^{0}C in a rotary shaking incubator overnight. 1 to 3 ml bacterial cells was pelleted by centrifugation at 13,000 g for 30 seconds in a microfuge. The pellet was resuspended in 200 µl Resuspension Buffer (50 mM Tris-HCl, pH 7.5; 10 mM EDTA, 100µg/ml RNase A). 200 µl of Lysis Buffer (0.2M NaOH, 1% SDS) was added to lyse the cells. 200 µl of Neutralization Buffer (2.55M KOAc, pH 4.8) was added. The mixture was centrifuged at 13,000 g for 5 minutes. The supernatant was transferred to a fresh 1.5ml microfuge and 1 ml Magic Minipreps DNA Purification Resin was added. A Magic Minipreps Column was attached to a 3 ml syringe barrel. Then the supernatant/resin mixture was added into the barrel and pushed gently into the column. The column was washed with 2 ml Column Wash Solution (100 mM NaCl; 10 mM Tris-HCl, pH 7.5; 2.5 mM EDTA, 50% EtOH). The column was centrifuged for 30 seconds in a microcentrifuge to remove the last trace of solution. It was then attached to a fresh microfuge and 50 µl of pre-heated to 65^{0}C water was added to elute the DNA. The DNA was collected by centrifugating the column at 13,000 g for 30 seconds.

2.14.2 Megapreparation of Plasmid using Qiagen-tip 100. A single bacterial colony was inoculated into 100 ml LB medium containing appropriate antibiotic. The

culture was incubated at 37^{0}C overnight in a rotary incubator. The bacterial cells were collected by centrifugation at 10,000 g for 15 minutes at 4^{0}C. The bacterial pellet was resuspended in 4 ml Buffer P1. 4 ml Buffer P2 was added and the mixture was kept on ice for 15 minutes. It was centrifuged at 13,000 g for 30 minutes at 4^{0}C. The supernatant was transferred to a fresh tube. A Qiagen-Tip 100 (Qiagen Inc.) was equilibrated with 4ml QBT, and the top was allowed to empty by gravity flow. The supernatant collected was loaded onto the tip. After the tip was emptied, it was washed with 10 ml QC buffer twice. The plasmid DNA adsorbed onto the tip was eluted by 5 ml QF buffer. The eluted plasmid was recovered by adding 0.7 volume of isopropanol. The mixture was kept at room temperature for 30 minutes and then centrifuged at 13,000 g for 15 minutes at 4^{0}C. The pellet was washed with 70% ethanol and recovered by centrifugation again. It was air-dried and dissolved in an appropriate volume of water. (The constituents of buffers P1, P2, P3, QBT, QC and QF are mentioned in Section 2.1.4).

2.15. Single-Stranded DNA Preparation

2.15.1 Transfection. The ligation mixture was transformed (Section 2.11) into *E.coli* strain M13mp18 and mp19 replicative form vectors. The culture with an aliquot of an extra 100 µl overnight cells was added into 4 ml H-top agar prewarmed to 45^{0}C with 80 µl 20 mg/ml Xgal and 0.01M IPTG. After the agar has set, it was incubated at 37^{0}C overnight.

2.15.2 Single-Stranded DNA Isolation. A plaque was picked with a toothpick and inoculated into 3 ml 2TY medium containing 1:100 dilution of overnight cells and appropiate antibiotics. It was then incubated at 37^{0}C for 6-8 hours with vigorous agitation. 1.5 ml culture was centrifuged at 13,000 g for 10 minutes. The supernatant was transferred to a fresh microfuge and centrifuged again to remove the bacterial cells. The supernatant was then added with 0.1 volume of 3M ammonium acetate (pH 5.5) and 200

μl 20% (w/v) PEG 6000. It was kept on ice for 30 minutes. After that, the phage was pelleted by centrifugation at 13,000 g for 15 minutes at 4^0C. The pellet was then dissolved in 200 ul TE and extracted with phenol chloroform, and once with chloroform extraction (Secion 2.3). The ss DNA was precipitated with absolute ethanol (Section 2.4).

2.16. DNA Sequencing

2.16.1 Plasmid Sequencing using T7 Sequencing Kit. Dideoxy-sequencing of plasmid was carried out by the T7 Sequencing kit from Pharmacia. 1-2 μg plasmid DNA was denatured with 8 μl of 2M NaOH in 40 μl volume at 37^0C for 1 hour. The denatured template was recovered by ethanol precipitation (Section 2.4) and dissolved in 10 μl cold water. 2 μl of annealing buffer and 2 μl of primer (5 μg/ml) was added to the template. The mixture was incubated at 37^0C for 20 minutes and then at room temperature for 10 minutes. 3 μl of Labelling Mix A, 1 μl of α-[^{35}S]dATP (10mCi/ml, Amersham), 1.6 μl of Enzyme Dilution Buffer and 0.5 units of T7 DNA Polymerase were added to the mixture. It was incubated at room temperature for 5 minutes. During the incubation, 2.5 μl of A-, G-, C-, T-, Mix Short was added to four labelled 1.5 ml microfuge and they were pre-warmed at 43^0C. After incubation, 4.5 μl of the reaction premix was added to each of the four pre-warmed Short Mix. The terminaton reaction was taken place at 43^0C for 5 minutes. Then they were stopped by adding 5 μl Stop Solution. The samples were then kept at -20^0C before use.

2.16.2 Cycle Sequencing from PCR Products. Direct sequencing of PCR products was performed using SequiTherm^{TM} Cycle Sequencing Kits from Epicentre Technologies. The protocol described involved internal labelling with α-labelled nucleotides. 15 pmole of unlabelled primer, 1 μl of α-[^{35}S]-dATP (10 mCi/ml, Amersham), 2.5 μl 10X Sequencing Buffer, 500 fmoles of DNA template were made up to 16 μl with deionized water. 1 μl of

5 units SequiTherm DNA Polymerase was added. 4 µl of the reaction premix was added to 4 microfuges each with 2 µl of ddATP, ddGCP, ddCTP, ddTCP Termination Mix respectively. They were overlaid with a drop of mineral oil. The reaction was heated at 95^0C for 5 minutes and then subject to 30 cycles of 95^0C 30 seconds, 1 minute at 70^0C in a thermocycler (Thermolyne or MJ PTC-100). It was stopped by adding 3 µl Stop Buffer and kept at -20^0C before use.

2.16.3 Cycle Sequencing from PCR Products or Plasmid. The method described involved end-labelled primer. 5-10 pmole primer was labelled in 10 µl volume containing 2µl γ-[^{32}P or ^{33}P]-ATP (10mCi/ml, Amersham), 1 µl of 10X polynucleotide kinase buffer and 1 unit of T4 polynucleotide kinase at 37^0C for 1 hour. The following protocol was described for USB Thermo Sequenase cycle sequencing kit from Amersham. 100ng of single-stranded DNA or 200 ng of plasmid DNA, 2 µl Reaction Buffer, 1 µl Labelled primer, 2 µl of Thermo Sequenase DNA polymerase were mixed and made up to 17.5 µl with deionized water. 4 µl of the premix was added to 4 microfuges with 4 µl of ddATP, ddGTP, ddCTP, ddTTP Termination Mix respectively. They were overlaid with a drop of mineral oil. The reaction was heated at 95^0C for 5 minutes and then subject to 50 cycles of 30 seconds at 95^0C, 30 seconds at 55^0C, 1 minute at 72^0C in a thermocycler (Thermolyne). It was stopped by adding 4 µl Stop Solution and kept at -20^0C before use.

2.16.4 DNA Sequencing Electrophoresis.

A Sequi-Gen® Nucleic Acid Sequencing Cell (Bio-Rad) was used.

2.16.4.1 Preparation of 6% Polyacrylamide Gel Soultion. 29.4 g of urea, 10.5 ml of 40% acrylamide solution and 7 ml 10X TBE were mixed. It was made up to 70 ml with distilled water.

2.16.4.2 Gel Casting. The glass surfaces in contact with the gel of the casting unit were cleaned with distilled water, 70% ethanol and acetone thoroughly. One of the glass surface was wiped with repellent, dimethyldichlorosilane solution 2% (w/v) in 1,1,1-tricholoethane. A pair of 0.4mm thick spacers was placed between the cleaned glass surfaces. The cell was then assembled by the clamps provided by the supplier. 10 ml of 6% acrylamide gel soultion, 125 µl of 10% ammonium persulfate and 50 µl TEMED were mixed to seal the bottom of the cell. The mixture was poured onto a sealing strip in a casting tray. The cell was placed on the top of the sealing strip.

After the bottom of the cell was sealed, it was placed in an inclined position of about 20 degrees. 360 µl of 10% ammonium persulfate and 35 µl TEMED were added to the remaining 60 ml 6% acrylamide gel solution. It was then poured immediately into the gap between the glass plates of the casting unit. A 0.4mm thick shark-tooth comb was inserted into the gap between the glass plates with its flat side facing inward. The gel was allowed to set for an hour.

2.16.4.3 Electrophoresis of Sequencing Gel. After the gel had set, the comb was removed and it was inserted again with its toothed side pointing inward. The casting unit was then assembled onto the gel tank. The upper and lower buffer reservoirs were filled with 1X TBE. The gel was pre-run at a constant power of 50W for 30 minutes. Before loading samples, they were heated in boiling water for 2 minutes. 3 µl of sample was loaded into the well using a sequencing pipette (Drummond). The gel was run at a constant power of 50W until the dyes reached the desired position. The casting unit was dissembled and the gel was blotted onto a sheet of 3MM Whatman filter paper. It was covered with a piece of Saran wrap and dried in a gel dryer (Slab Gel Dryer SGD2000) for 1.5 hours. Autoradiography was performed to detect the sequencing bands.

2.16.4.5. Autoradiography. Kodak X-OMAT AR diagnostic film or Fuji RX medical X ray film was used for detection. Film was exposed in a Kodak X-Omatic cassette at room temperature (for ^{35}S isotope) or -70^{0}C (for ^{32}P or ^{33}P isotopes) from one

day to one week depended on the signal of the gel. After exposure, the film was developed with Kodak X-ray developer for 5 minutes, rinsed in running water and then fixed with Kodak X-ray fixer for 5 minutes. It was then rinsed with running water for 5 minutes.

Chapter 3 Studies of *Panax* species by Random-Primed PCRs

Chapter 3 Studies of *Panax* Species by Random-Primed PCRs

3.1 Introduction

Ginseng (Fig.3.1) belongs to the genus *Panax* in the family of Araliaceae. Oriental ginseng (*P. ginseng*) is grown in the Northeast China, Korea and Japan. It has been used for many years in the Orient as a tonic, prophylatic and anti-aging agent. American ginseng (*P. quinquefolius*) (Fig.3.2), on the other hand, is mainly cultivated in Wisconsin, USA and British Columbia, Canada. It was introduced to the Orient in the past century. In the traditional Chinese medicine, it is said to possess the "cooling" effect to clear 'heat' inside the body.

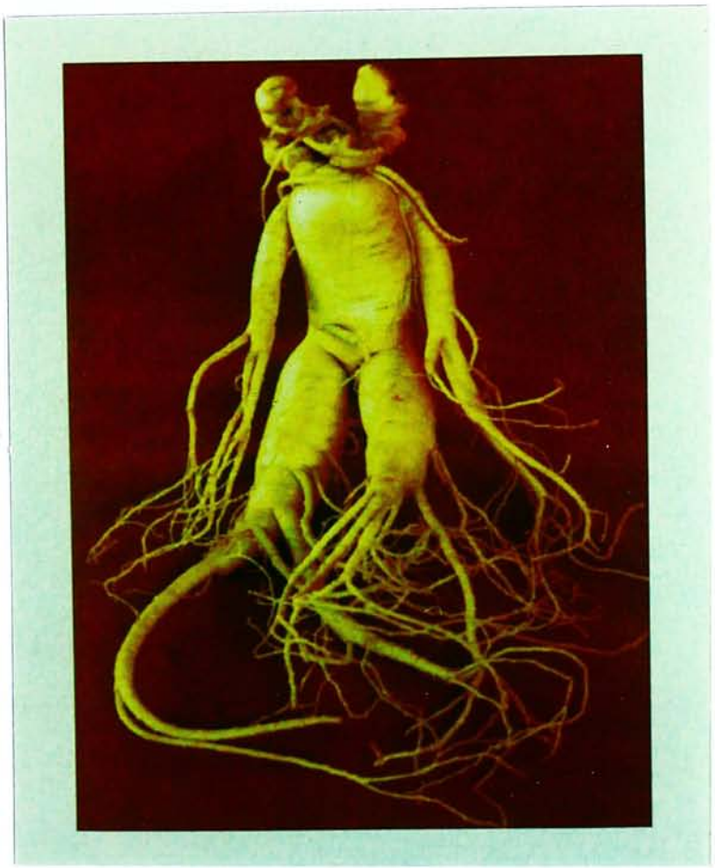

Fig.3.1: The root of ginseng. Ginseng belongs to the genus *Panax* which means man-like. It is a superior herb in the Chinese medicinal herbs and has been used for thousands of years.

Fig.3.2: The root of *P. quinquefolius* (American ginseng).

Ginseng is a big trade in Hong Kong. In 1995, more than 3 million kilograms of American and Oriental ginsengs worth HK$1500 million were imported. The retail price of cultivated American ginseng is usually much more expensive than that of cultivated Oriental ginsengs produced in China. It prompts the wide-spread practice of disguising Oriental ginsengs as American ginsengs by some merchants. These two species have different pharmacological actions. Adulterants including *Mirabilis jalapa* L, *Phytolacca acinosa* Roxb and *Platycodon grandiflorum* (Fig.3.3) are sold as Oriental ginsengs on the market.

(a)

(b)

(c)

Fig.3.3: The adulterants of ginsengs. (a). *P. grandiflorum*. (b). *P. acinosa* Roxb. (c) *M. jalapa*. They are disguised as *P. ginseng*. The last two plants are poisonous.

In traditional Chinese medicine, *P. grandiflorum* is for eliminating phlegm and ventilating the lungs. *M. jalapba* is for treating diarrhea, dissolving the stagnant blood and maintaining the blood circulation. *P. acinosa* is for eliminating water in edema. Roots of the last two species are poisonous and *P. acinosa* has been responsible for several reported cases of "ginseng"-poisoning in China.

Besides *P. quinquefolius* and *P. ginseng*, another important medicinal plant in the same genus is *P. notoginseng* (Sanchi) (Fig.3.4). It is used as a hematostatic for hemorrage. *Curcuma zedoara* has been found as an adulterant of Sanchi (Fig.3.5).

Fig.3.4: The root of *P. notoginseng* (Sanchi). It is of conical-shape and is used as a hematostatic for hemmorrage.

Fig.3.5: *C. zedoara,* the adulterant of Sanchi. The root is also of conical-shape.

With the advancement in molecular studies, genomic constitution can be used to differentiate Chinese herbs. Our group has used two techniques: Randomly Amplified Polymorphic DNA (RAPD) and Arbitarily Primed-Polymerase Chain Reaction (AP-PCR) to establish DNA fingerprints of both *P. quinquefolius* and *P. ginseng* as well as their adulterants (Shaw & But, 1995). In this study, six *Panax* species were collected and used in the DNA fingerprinting. The relatedness of these six *Panax* species was also estimated.

3.2 Materials and Methods

3.2.1 Plant Materials

Dried or fresh roots of *Panax* species were obtained from various sources as shown in Table 3.1.

Samples	Origins
P. quinquefolius, dried root aged 3	Canada
P. ginseng, dried root	China
P. notoginseng (Burk),F.H.Chen, fresh root	China
P. japonicus major, fresh root	China
P. japonicus, fresh root	Japan
P. trifolius, fresh root	USA

Table 3.1. The origin of the six *Panax* species.

Samples were identified by Dr. P.P.H. But and stored in the museum of the Chinese Medicinal Material Research Centre.

3.2.2 DNA Extraction and Random-Primed PCRs

The procedure of DNA extraction is described in Section 2.2. AP-PCR and RAPD were performed according to Section 2.6. 100-200 ng and 25 ng of DNA were used in AP-PCR and RAPD, respectively.

3.2.3 Data Analysis

The bands were recorded as 0 (absent) or 1 (present). The relatedness of the various *Panax* species was estimated by calculating the Similarity Index (S.I.), the fraction of shared fragments between two samples, using the formula: S.I.=2Nxy/Nx+Ny, where Nxy is the number of bands shared between two samples. Nx and Ny are the number of bands present in samples X and Y, respectively (Nei & Li, 1979). The relationship was then illustrated by plotting the average genetic distance, F=1-S.I. from different DNA

fingerprints using UPGMA clustering in the software PHYLIP (Felsenstein, 1993). This method forms group by successively pairing similar molecular phenotypes according to the magnitude of their observed distances (Sneath & Sokal, 1973). Only those clear and distinctive bands were scored. Vague and low intensity bands were excluded in the scoring process.

3.3 Results and Discussion

3.3.1 DNA Isolation

The samples used were fresh or dried roots. High molecular weight DNA were isolated from most *Panax* species except *P. trifolius* (Fig.3.6). Nevertheless, judging from the gel, DNA from *P. trifolius* was of sufficiently high molecular weight for DNA fingerprinting.

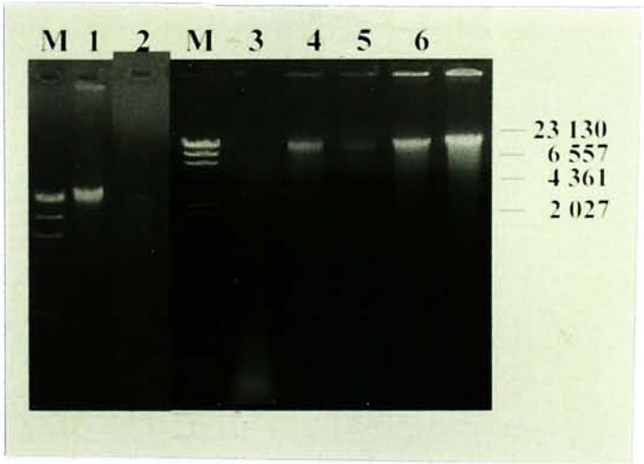

Fig.3.6: DNA extracted from the six *Panax* species. Lane 1: *P.quinquefolius*, Lane 2: *P.ginseng*, Lane 3: *P. trifolius*, Lane 4: *P. japonicus* major, Lane 5: *P. notoginseng*, Lane 6: *P. japonicus*. M: λ*Hin*dIII digested marker.

3.3.2 DNA Fingerprinting

Figs.3.7 and 3.8 show the DNA fingerprints of the six *Panax* species by AP-PCR using primers Seq2 and TCS backward, respectively. Obviously, distinctive bands existed for differentiation of the six *Panax* species.

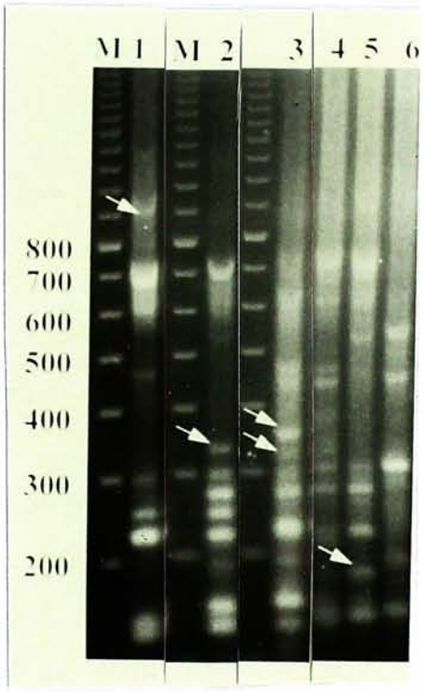

Fig.3.7: DNA fingerprint of the six *Panax* species. It was generated by AP-PCR using Seq2:5'CTG GTC AAG GCA CAA GAG AT3'. Lane: 1: *P. quinquefolius*. Lane 2: *P. ginseng*, Lane 3: *P. notoginseng*, Lane 4: *P. japonicus*, Lane 5: *P. japonicus* major, Lane 6: *P. trifolius*. M: 100 bp ladder marker. Arrows indicate distinctive bands of a particular species.

When Seq2 was used as primer, the bands produced ranged from 100 to 1 000 bp. Although the range was quite large, most of the bands were around 200 to 300 bp and they were shared between two or even all the species.

The genetic distances are summarized in Table 3.2.

	P.Q	P.G	P.N	P.J	P.J.M
P.G	0.30				
P.N	0.39	0.23			
P.J	0.45	0.44	0.36		
P.J.M	0.30	0.30	0.30	0.36	
P.T	0.63	0.68	0.58	0.56	0.50

Table 3.2: The genetic distance (Section 3.2.3) of the six *Panax* species calculated from Seq2. P.Q: *P. quinquefolius*, P.G: *P. ginseng*, P.N: *P. notoginseng*, P.J: *P. japonicus*, P.J.M: *P. japonicus* major, P.T: *P. trifolius*.

The relationship is illustrated as follows:

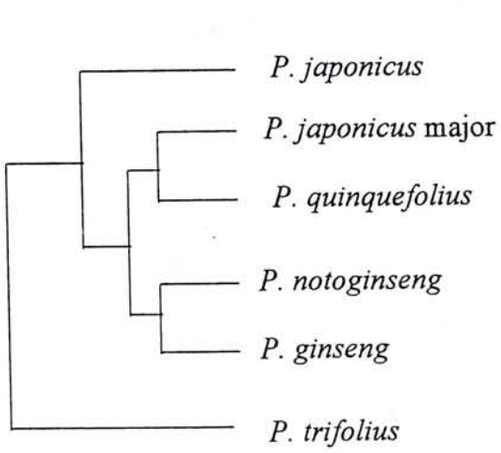

Fig.3.8: A phylogenetic tree showing the relationship among the six *Panax* species using primer Seq2.

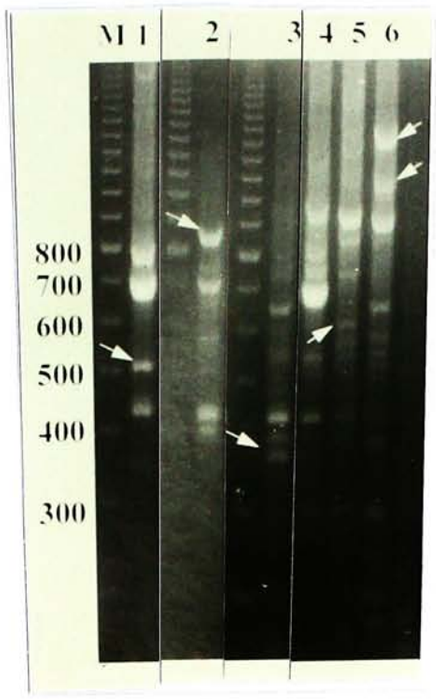

Fig.3.9: DNA fingerprint of the six *Panax* species. It was generated by AP-PCR using TCS backward: 5'GGT GGA TCC CTA AGC ATC AAC ATT GGT3'. Lane: 1: *P. quinquefolius*. Lane 2: *P. ginseng*, Lane 3: *P. notoginseng*, Lane 4: *P. japonicus*, Lane 5: *P. japonicus* major, Lane 6: *P. trifolius*. M: 100 bp ladder marker. Arrows indicate distinctive bands of a particular species.

When TCS backward was used as primer, the bands produced were in the range between 300 to 1 300 bp. A common band of 420 bp was found among most species except *P. trifolius;* while other were shared between two to four species. Almost all the six species had their own unique bands. A band of about 500 bp was present in *P. quinquefolius*. *P. ginseng* had a unique band of 830 bp; while *P. notoginseng* was characterized by the band of 380 bp. Two high molecular bands of 1 000 and 1 300 bp were observed in *P. trifolius.*

The genetic distances are summarized in Table 3.3.

	P.Q	P.G	P.N	P.J	P.J.M
P.G	0.17				
P.N	0.60	0.29			
P.J	0.50	0.57	0.53		
P.J.M	0.57	0.50	0.47	0.50	
P.T	0.89	0.62	0.58	0.81	0.48

Table 3.3: The genetic distance (Section 3.2.3) of the six *Panax* species calculated from TCS backward. P.Q: *P. quinquefolius*, P.G: *P. ginseng*, P.N: *P. notoginseng*, P.J: *P. japonicus*, P.J.M: *P. japonicus* major, P.T: *P. trifolius*.

The relationship is illustrated as follows:

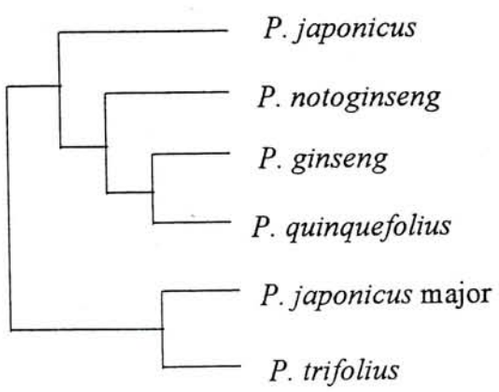

Fig.3.10: A phylogenetic tree showing the relationship among the six *Panax* species using primer TCS backward.

3.3.3 Relationship between the Six Panax Species

A consensus tree (Fig.3.11) was plotted by combining individual phylogenetic trees from the two primers. It showed that *P. quinquefolius, P. ginseng and P. notoginseng* belonged to the same group and the former two species were more closely related. This observation was consistent with the morphological or chemical analysis (Chen, 1994; Lang *et al*, 1993). Morphologically, both *P. quinquefolius* and *P. ginseng* have erect rhizomes and both are connected to a spindle-shaped fleshy root with branches, while the rhizome of *P. notoginseng* is also erect but it has a conical-shaped fleshy root. The rest of them do not have any erect rhizomes. *P. japonicus* and *P. japonicus* major have horizontal rhizomes. However, in *P. japonicus* the rhizomes has stout and short internodes but in *P. japonicus* major, it has elongated and enlarged nodes giving the appearance of a string of pearls (Fig. 3.12a and b). *P. trifolius* is recognized by its spherical fleshy root (Fig.3.12c). Furthermore, *P. quinquefolius, P. ginseng* and *P. notoginseng* mainly contain the drammarane type of ginsenosides (Fig.3.13a), while the rest contains oleanane type (Fig.3.13b).

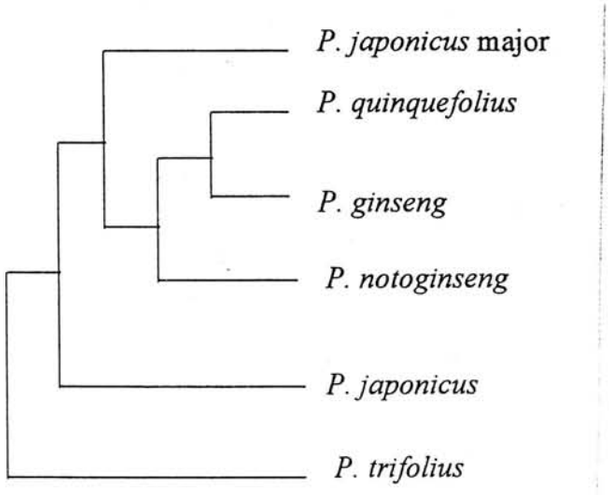

Fig.3.11: A consensus tree showing the relationship between the six *Panax* species. The genetic distances F=1-S.I. were plotted by UPGMA method in the PHYLIP program.

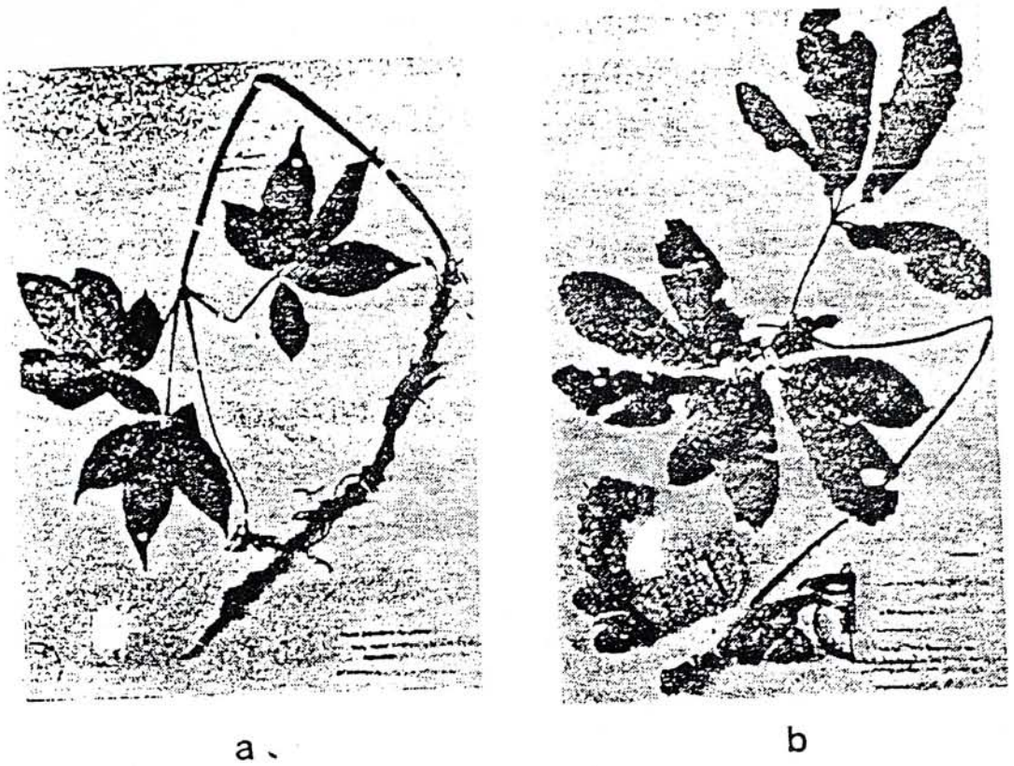

Fig.3.12. The morphology of the *Panax* species without erect rhizomes. (a) A flowering plant of *P. japonicus* showing a horizontal rhizome with stout and short internodes. (b) A flowering plant of *P. japonicus* major showing enlarged nodes that appears as a string of pearls. (c) A flowering plant of *P. trifolius* showing spherical root. (Hu, 1978)

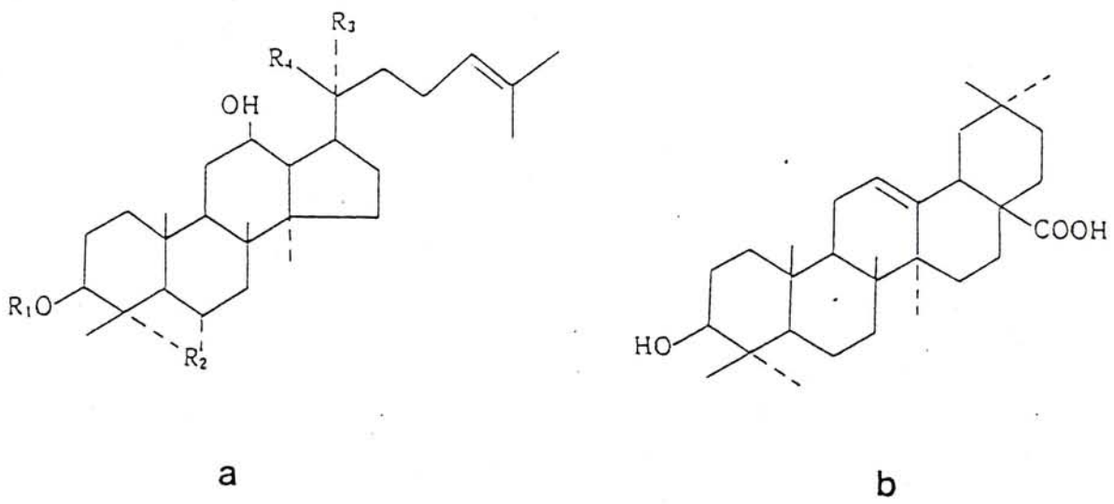

Fig.3.13: The structures of the (a) drammarane and (b) oleanane type of ginsenosides.

Chapter 4 Studies of *Acorus* by Random-Primed PCRs

Chapter 4 Studies of *Acorus* by Random-Primed PCRs

4.1. Introduction

Acorus (Fig.4.1) is a primal extant monocotyledon found in the West, Southwest or South of China. The root of this genus is used to treat mental confusion due to epilepsy or distention and distress in the epigastium.

Fig.4.1: The plant of an *Acorus* species.

The genus arose much controversial in systematic botany. It was originally grouped in the family Araceae. Nevertheless, morphological, anatomical, embryological characteristics (Grayum, 1987) as well as the sequence of ribulose-1,5-carboxylase large subunit (Duvall *et al*, 1993) have revealed that it does not have a close relationship with other plants in Araceae. Therefore, it is classified in an individual group called Acoraceae.

Systematists have tried to establish the relationship of individual *Acorus* species. However, traditional classification of *Acorus* species deserved particular comment as there are no distinctive characters. Four species are mentioned in the Flora Reipublicae Popularis Sinicae (Li, 1979). The identification is based on the leaves and length of buds. Recently, seven species and two varieties of *Acorus* have been identified around the world (Wu *et al*, 1994). Some botanists do not accept them as independent species as the identification is only based on the different morphological characters, or even on the odor of the root. The existing confusion indicates that morphological characters of the different plants are subtle and ambiguous. A more accurate and objective identification of *Acorus* species is needed

In this chapter, random-primed PCRs were adopted to resolve the ambiguity in *Acorus* identification.

4.2 Materials and Methods

4.2.1 Plant Materials. Nine samples of *Acorus* dried roots (Fig.4.2) were collected in the West of China and identified by Professor J. L. Wu. The samples were kept in the CMMRC for further reference.

Fig.4.2: The nine *Acorus* species studied. The roots of them are long, slender and brown with obvious internodes.

4.2.2 DNA Extraction and Random-Primed PCRs DNA extraction is described in Section 2.2. AP-PCR and RAPD were carried out as in Section 2.6. 10 ng of DNA was added in both random-primed PCRs. 1 and 0.5 unit of *Taq* polymerase were used in AP-PCR and RAPD, respectively.

4.3 Results and Discussion

4.3.1 Acorus DNA

The DNA extracted from the nine *Acorus* species is shown in Fig.4.3. In most of the samples, high molecular weight DNA was isolated.

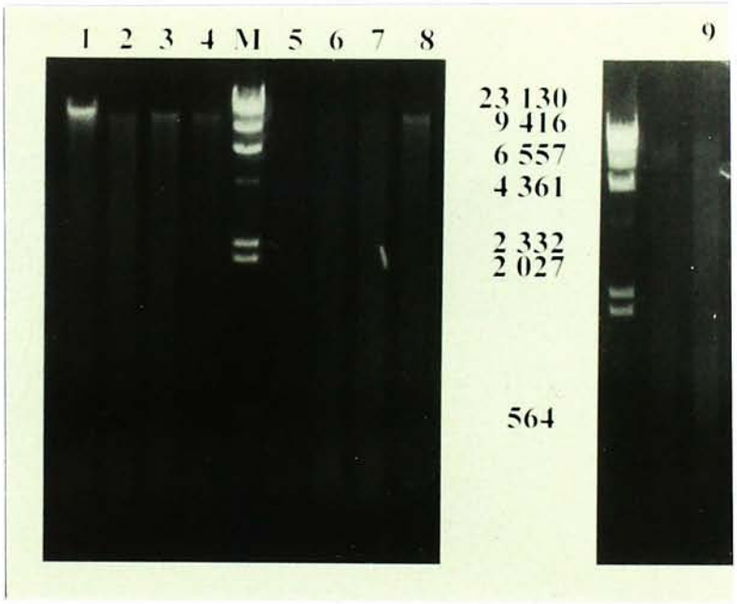

Fig.4.3: The DNA extracted from the *Acorus* species. Lane 1: *A. xiangyeus*, Lane 2: *A. latifolia*, Lane 3: *A. gramineus* var. flavo-marginatus, Lane 4: *A. gramineus*, Lane 5: *A. macrospadiceus*, Lane 6: *A. tatarinowii*, Lane 7: *A. calamus* L., Lane 8: *A. rumphianus*, Lane 9: *A. brevispathus*. M: λ*Hin*d III digested DNA as marker.

4.3.2 Reproducibility of Random-Primed PCRs

In DNA fingerprints study, reproducibility is always a concern. Several bands could not be reproduced even the same batch of DNA, reagents, primers as well as PCR machine were used. Polysaccharides, phenolic compounds or RNA might affect the reaction. The quality of DNA was determined by the ratio of O.D.260 nm/O.D.280 nm. The ratio ranged from 1.3 to 1.7. Further improvement was tried by purification of the DNA by CsCl gradient ultracentrifugation.

The effect of the different concentrations of DNA on the fingerprint in the range of 1 ng, 5 ng, 10 ng and 20 ng was studied. Fig. 4.4 shows the results of the DNA fingerprint generated from different DNA concentrations of two samples which had a low purity of DNA. The O.D. ratio of *A. latifolia* was 1.6 and that of *A. gramineus* was 1.4. In *A. latifolia*, the banding patterns were almost the same except two additional bands of 620 and 1 100 bp were produced in 5 ng DNA (Lane 2). In *A. gramineus*, the DNA profiles were nearly identical in the DNA concentrations of 1 and 5 ng (Lanes 5 and 6). An additional band of 530 bp was produced using 5 ng DNA. Fewer bands were generated in 10 ng DNA (Lane 7) and no bands was produced in 20 ng DNA (Lane 8). The last case might be due to the presence of too much contaminants that inhibited the reaction.

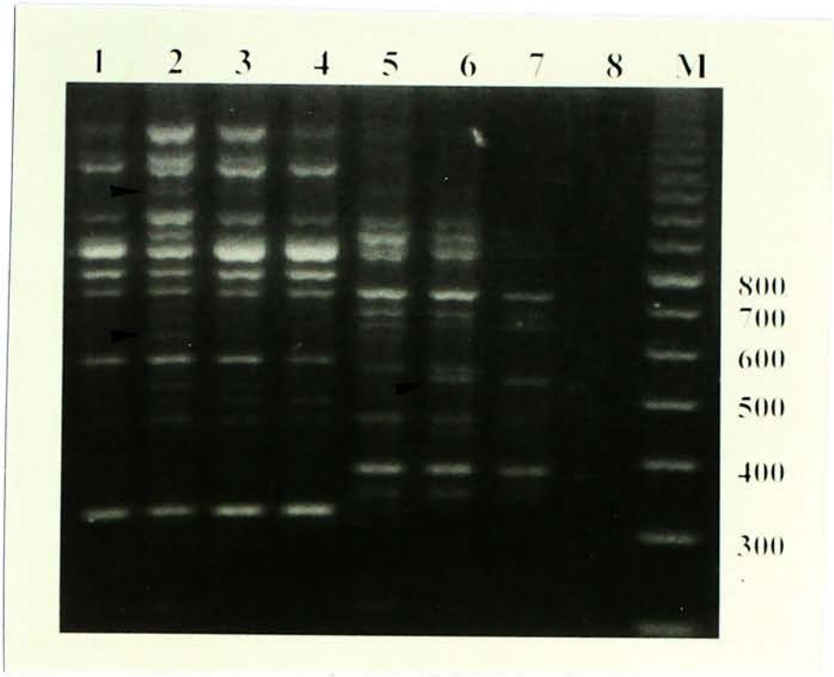

Fig.4.4: DNA profiles generated from different concentrations of two *Acorus* species. Lane 1: 1 ng of *A. latifolia*; Lane 2: 5 ng of *A. latifolia*; Lane 3: 10 ng of *A. latifolia*; Lane 4: 20 ng of *A. latifolia*; Lane 5: 1 ng of *A. gramineus*; Lane 6: 5 ng of *A. gramineus*; lane 7: 10 ng of *A. gramineus*; Lane 8: 20 ng of *A. gramineus*. M: 100bp ladder marker. OPAA-06: 5'GTG GGT GCC A3' was used to generate the fingerprint. Arrows indicate the additional bands produced.

We used primers that had higher reproducibility. Moreover, only those intense and reproducible bands that always appeared were used for identification and for phylogenetic studies. Vague bands were not included in the scoring process. Each primers was repeated at least three times to get the reproducible patterns. All the bands scored had more than 50% reproducibility.

4.3.3 DNA Fingerprinting

One hundred and six primers (Table 4.1) were tested on the species. Seven of them gave distinctive fingerprints. Figs.4.5 to 4.7 show the fingerprints generated from AP-PCR and Figs.4.8 to 4.11 are the fingerprints from RAPD. The nine samples of *Acorus* had different banding patterns. It suggested that they had different genomic constitutents and could be distinguished as nine individual species.

RAPD Primers	AP-PCR Primers
OPC-01 to OPC-20	Gal K
OPF-01 to OPF-20	M13 forward
OPAA-01 to OPAA-20	Seq 2
OPAM-01 to OPAM-20	TCS forward
OPAW-01 to OPAW-15	TCS backward
	primer#13
	primer#14
	primer#15
	primer#17
	primer#18
	primer#20

Table 4.1: The list of primers tested on the *Acorus* species. Sequences of the primers are listed in Appendix II.

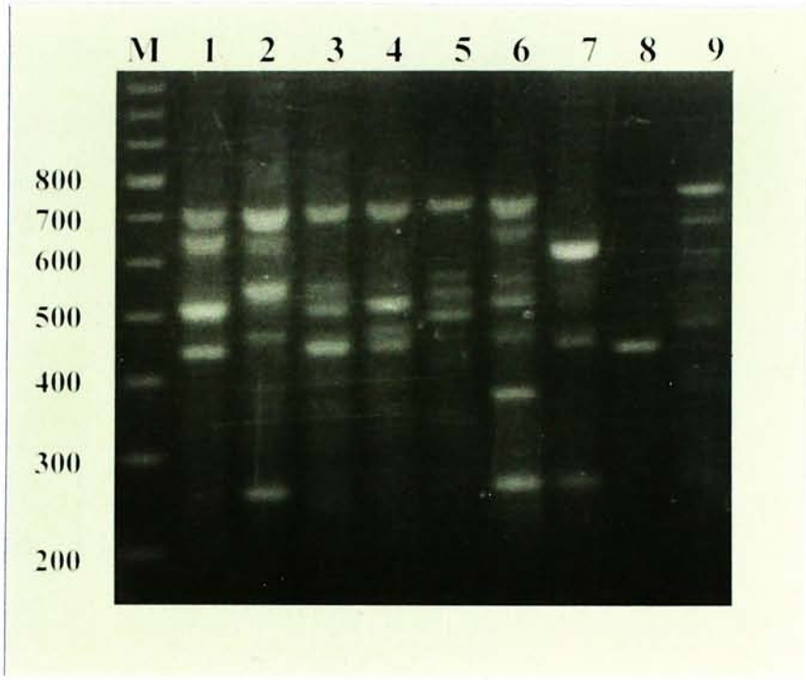

Fig.4.5: DNA fingerprint of nine *Acorus* species. It was generated by AP-PCR using primer#18:5'CAT CGG ATC CAC CAC GTC3'. Lane 1: *A. xiangyeus*, Lane 2: *A. latifolia*, Lane 3: *A. gramineus* var. flavo-marginatus, Lane 4: *A. gramineus*, Lane 5: *A. macrospadiceus*, Lane 6: *A. tatarinowii*, Lane 7: *A. calamus* L., Lane 8: *A. rumphianus*, Lane 9: *A. brevispathus*. M: 100 bp ladder marker.

The bands produced ranged from 200 to 700 bp. Most of the bands were of 400 to 600 bp. A band of about 700 bp was shared among most of the species except *A. calamus* which was characterized by the intense band of 600 bp. The fingerprints of *A. gramineus* var. flavo-marginatus and *A. gramineus* were identical and that *of A. xiangyeus* was similar to them. On the other hand, the DNA profiles of *A. latifolia* and *A. tatarinowii* were very similar.

The genetic distances are tabulated as follows:

	A.X	A.L	A.G.V	A.G	A.M	A.T	A.C	A.R
A.L	0.11							
A.G.V	0.11	0.25						
A.G	0.20	0.33	0.11					
A.M	0.56	0.50	0.50	0.33				
A.T	0.17	0.27	0.27	0.33	0.64			
A.C	0.50	0.71	0.43	0.50	1.00	0.60		
A.R	0.50	0.71	0.43	0.50	1.00	0.60	0.00	
A.B	0.00	0.11	0.11	0.20	0.56	0.17	0.50	0.50

Table 4.2: The genetic distances, F=1-S.I (see section 3.2.3), of nine *Acorus* species calculated from primer#18. A.X: *A. xiangyeus*, A.L: *A. latifolius*, A.G.V: *A. gramineus* var. flavo-marginatus, A.G: *A. gramineus*, A.M: *A. macrospadiceus*, A.T: *A. tatarinowii*, A.C: *A. calamus* L., A.R: *A. rumphianus*, A.B.: *A. brevispathus*

Using primer#18, the genetic distances between the pairs of *A. calamus* and A. *rumphianus*; *A. xiangyeus* and *A. brevispathus* were zero. That meant they were identical from the DNA fingerprint. On the other hand, the genetic distances of *A. macrospadiceus* and *A. calamus* as well as *A. rumphianus* were equal to one. It indicated that they were distantly related.

The relationship is illustrated as follows:

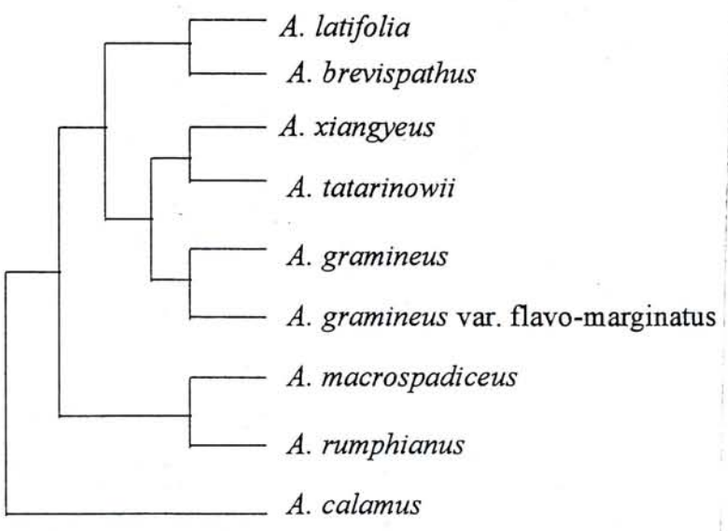

Fig.4.6: A phylogenetic tree showing the relationship among the nine *Acorus* species using primer #18

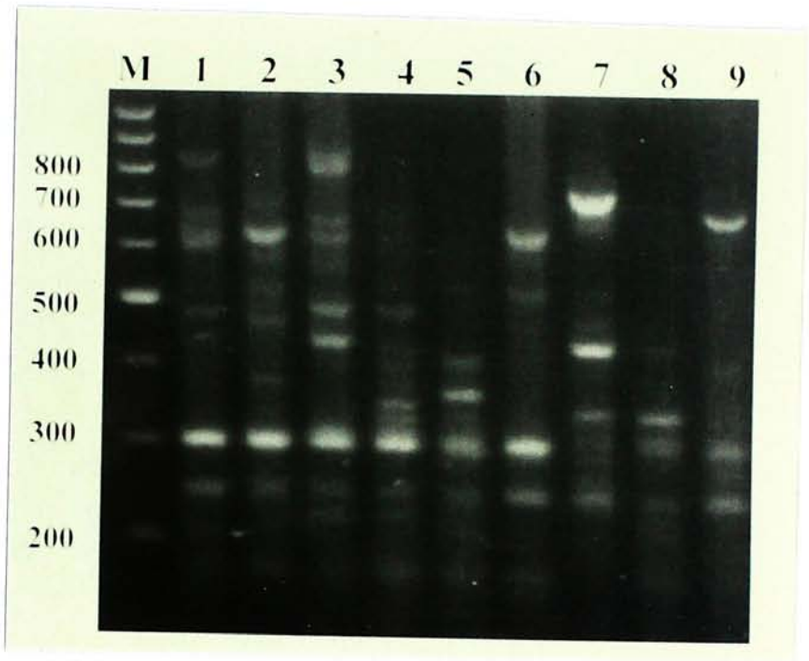

Fig.4.7: DNA fingerprint of nine *Acorus* species. It was generated by AP-PCR using M13F: 5'CGC CAG GGT TTT CCC AGT CAC GAC3'. Lane 1: *A. xiangyeus*, Lane 2: *A. latifolia*, Lane 3: *A. gramineus* var. flavo-marginatus, Lane 4: *A. gramineus*, Lane 5: *A. macrospadiceus*, Lane 6: *A. tatarinowii*, Lane 7: *A. calamus* L., Lane 8: *A. rumphianus*, Lane 9: *A. brevispathus*. M: 100 bp ladder marker.

The bands produced ranged from 200 to 800 bp. A prominent band of 300 bp was shared between most species except *A. calamus*. Another band of 250 bp was found among all species. The banding patterns of *A. xiangyeus, A. gramineus* var. flavo-marginatus and *A. latifolia* and *A. tatarinowii* were very similar.

The genetic distances are tabulated as follows:

	A.X	A.L	A.G.V	A.G	A.M	A.T	A.C	A.R
A.L	0.25							
A.G.V	0.40	0.25						
A.G	0.50	0.33	0.25					
A.M	0.75	0.67	0.75	0.67				
A.T	0.25	0.00	0.50	0.33	0.67			
A.C	0.60	0.50	0.40	0.50	0.75	0.50		
A.R	0.71	0.60	0.71	0.60	0.60	0.60	0.43	
A.B	0.43	0.20	0.43	0.20	0.60	0.20	0.43	0.50

Table 4.3: The genetic distances, F=1-S.I (see section 3.2.3), of nine *Acorus* species calculated form M13 forward. A.X: *A. xiangyeus*, A.L: *A. latifolius*, A.G.V: *A. gramineus* var. flavo-marginatus, A.G: *A. gramineus*, A.M: *A. macrospadiceus*, A.T: *A. tatarinowii*, A.C: *A. calamus* L., A.R: *A. rumphianus*, A.B.: *A. brevispathus*

The relationship is illustrated as follows:

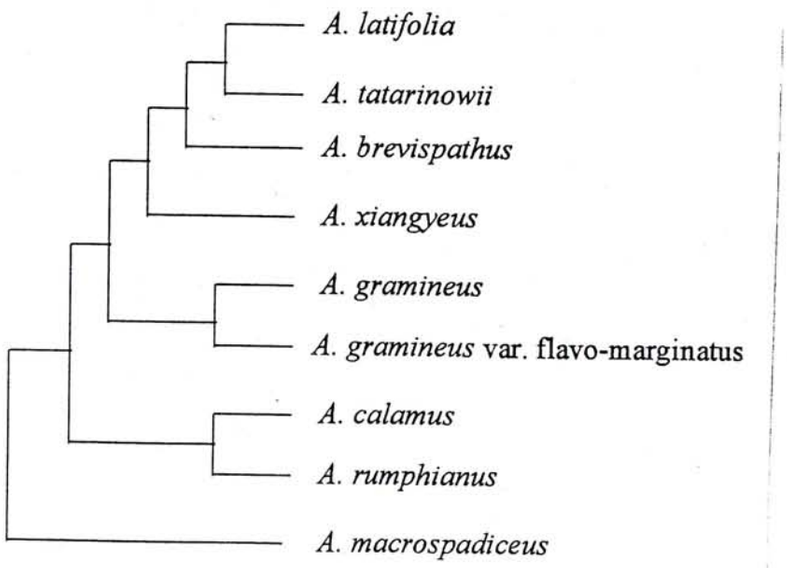

Fig.4.8: A phylogenetic tree showing the relationship among the nine *Acorus* species using M13 forward primer.

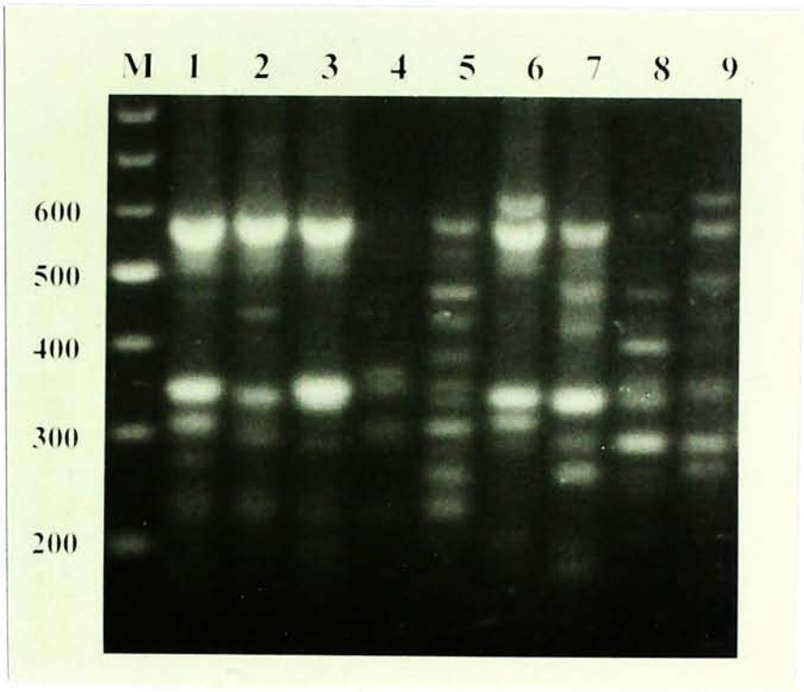

Fig.4.9: DNA fingerprint of nine *Acorus* species. It was generated by AP-PCR using Seq2: 5'CTG GTC AAG GCA CAA GAG AT3'. Lane 1: *A. xiangyeus*, Lane 2: *A. latifolia*, Lane 3: *A. gramineus* var. flavo-marginatus, Lane 4: *A. gramineus*, Lane 5: *A. macrospadiceus*, Lane 6: *A. tatarinowii*, Lane 7: *A. calamus* L., Lane 8: *A. rumphianus*, Lane 9: *A. brevispathus*. M: 100 bp ladder marker.

The bands produced ranged from 200 to 600 bp. A prominent band of 550 bp was shared among most of the species except *A. gramineus* and *A. rumphianus*. The patterns of *A. xiangyeus*, *A. latifolia*, *A. gramineus* var. flavo-marginatus, *A. tatarinowii* and *A. calamus* were almost the same with the intense bands of 550 and 350 bp.

The genetic distances are tabulated as follows:

	A.X	A.L	A.G.V	A.G	A.M	A.T	A.C	A.R
A.L	0.00							
A.G.V	0.14	0.14						
A.G	0.33	0.33	0.50					
A.M	0.33	0.33	0.25	0.43				
A.T	0.25	0.25	0.14	0.33	0.33			
A.C	0.43	0.43	0.33	0.20	0.25	0.43		
A.R	0.50	0.50	0.43	0.67	0.33	0.50	0.43	
A.B	0.25	0.25	0.14	0.33	0.11	0.25	0.14	0.25

Table 4.4: The average genetic distances, F=1-S.I (see section 3.2.3), of nine *Acorus* species calculated from Seq2. A.X: *A. xiangyeus*, A.L: *A. latifolius*, A.G.V: *A. gramineus* var. flavo-marginatus, A.G: *A. gramineus*, A.M: *A. macrospadiceus*, A.T: *A. tatarinowii*, A.C: *A. calamus* L., A.R: *A. rumphianus*, A.B.: *A. brevispathus*

Using primer Seq2, *A. xiangyeus* and *A. latifolia* were shown to be the most closely related species. Other species were quite similar as their genetic distances ranged from 0.11 to 0.67.

The relationship is illustrated as follows:

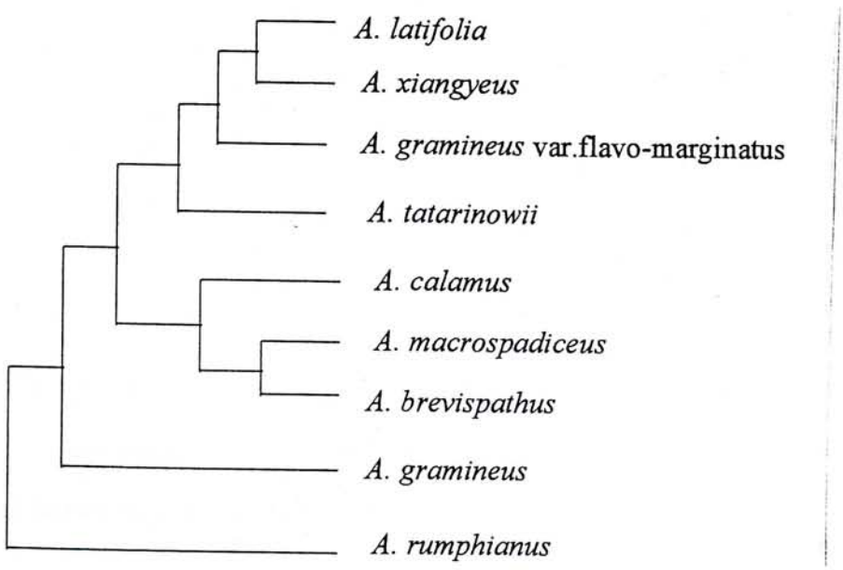

Fig.4.10: A phylogenetic tree showing the relationship among the nine *Acorus* species using primer Seq2.

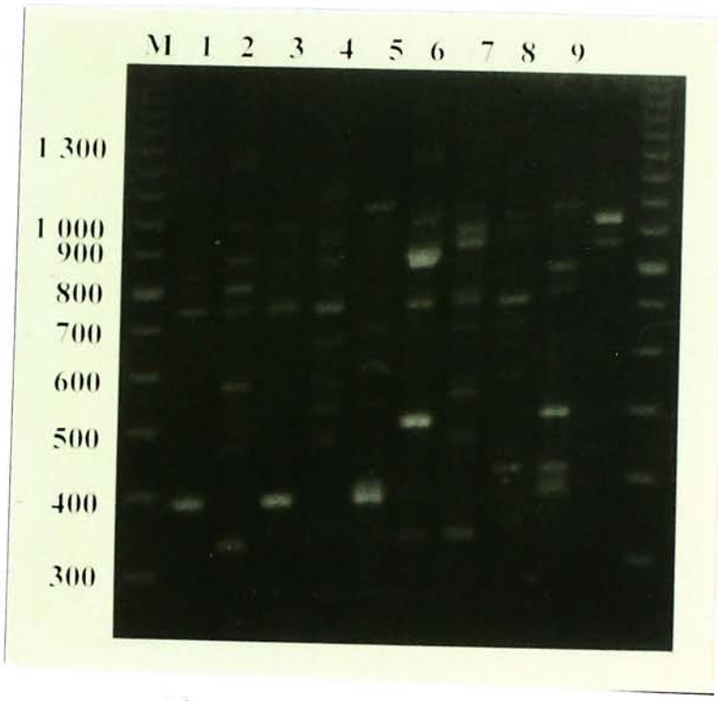

Fig.4.11: DNA fingerprint of nine *Acorus* species. It was generated by RAPD using OPAA-06: 5' GTGGGTGCCA 3'. Lane 1: *A. xiangyeus*, Lane 2: *A. latifolia*, Lane 3: *A. gramineus* var. flavo-marginatus, Lane 4: *A. gramineus*, Lane 5: *A. macrospadiceus*, Lane 6: *A. tatarinowii*, Lane 7: *A. calamus* L., Lane 8: *A. rumphianus*, Lane 9: *A. brevispathus*. M: 100 bp ladder marker.

The bands produced ranged from 300 to 1 100 bp. A band of about 750 bp was shared among most species except *A. macrospadiceus* and *A. brevispathus*. Some other bands were shared between individual species.

The genetic distances are tabulated as follows:

	A.X	A.L	A.G.V	A.G	A.M	A.T	A.C	A.R
A.L	0.45							
A.G.V	0.25	0.56						
A.G	0.56	0.40	0.43					
A.M	0.71	**1.00**	0.60	**1.00**				
A.T	0.56	0.40	0.43	0.75	**1.00**			
A.C	0.69	0.43	0.82	0.83	0.80	0.67		
A.R	0.75	0.78	0.67	0.71	**1.00**	0.71	0.82	
A.B	0.78	0.80	0.71	**1.00**	0.67	0.75	0.83	0.71

Table 4.5: The genetic distances, F=1-S.I (see section 3.2.3), of nine *Acorus* species calculated from OPAA-06. A.X: *A. xiangyeus*, A.L: *A. latifolius*, A.G.V: *A. gramineus* var. flavo-marginatus, A.G: *A. gramineus*, A.M: *A. macrospadiceus*, A.T: *A. tatarinowii*, A.C: *A. calamus* L., A.R: *A. rumphianus*, A.B.: *A. brevispathus*

Using primer OPAA-06, five pairs of species (highlighted) were distantly related. The genetic distances of the majority species were relatively high. It implied that they were not closely related.

The relationship is illustrated as follows:

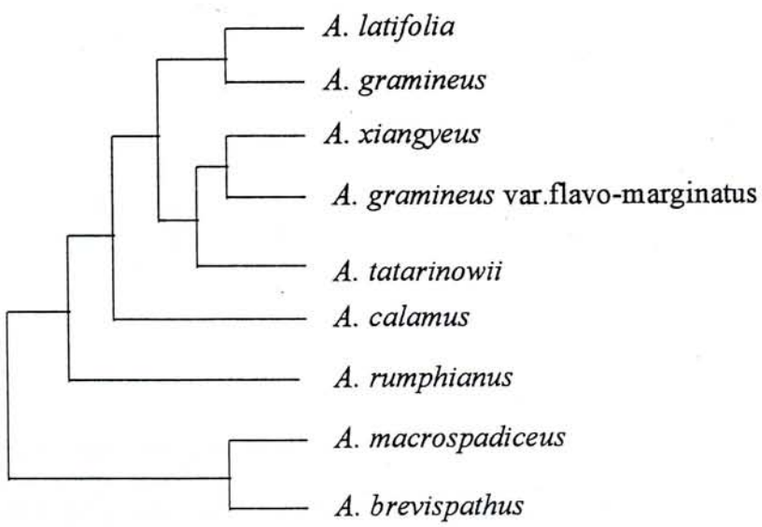

Fig.4.12: A phylogenetic tree showing the relationship among the nine *Acorus* species using primer OPAA-06.

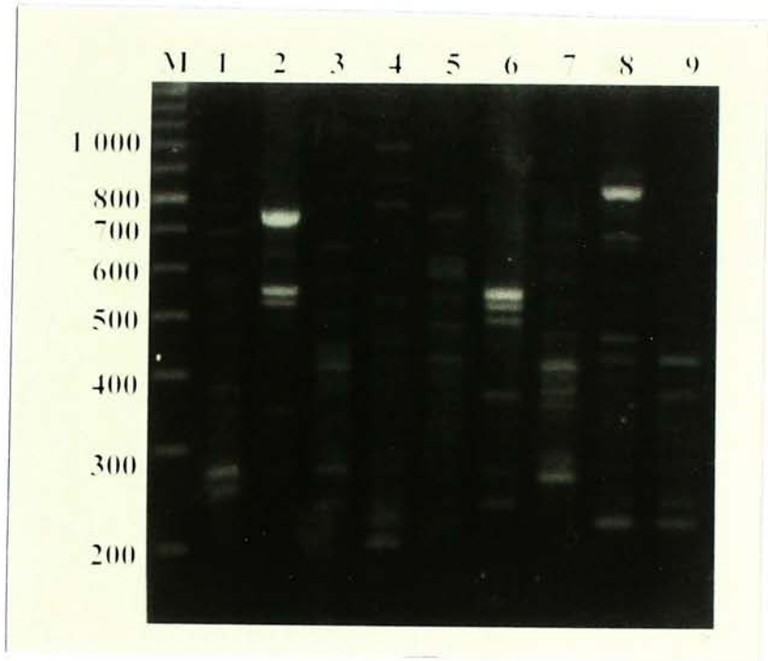

Fig.4.13: DNA fingerprint of nine *Acorus* species. It was generated by RAPD using OPAW-04: 5'AGG AGC GAC A3'. Lane 1: *A. xiangyeus*, Lane 2: *A. latifolia*, Lane 3: *A. gramineus* var. flavo-marginatus, Lane 4: *A. gramineus*, Lane 5: *A. macrospadiceus*, Lane 6: *A. tatarinowii*, Lane 7: *A. calamus* L., Lane 8: *A. rumphianus*, Lane 9: *A. brevispathus*. M: 100 bp ladder marker.

The bands produced ranged from 200 to 1 000 bp and they were scattered around. Common bands were only shared between two or among a few species but not all. The intensity of most of the bands were weak. Repeated reactions were unable to optimize the intensity.

The genetic distances are tabulated as follows:

	A.X	A.L	A.G.V	A.G	A.M	A.T	A.C	A.R
A.L	0.45							
A.G.V	0.65	0.82						
A.G	0.63	1.00	0.64					
A.M	0.75	1.00	0.64	0.60				
A.T	0.41	0.64	0.83	0.64	0.81			
A.C	0.65	1.00	0.50	0.45	0.45	0.67		
A.R	0.60	0.78	0.80	0.78	0.56	1.00	0.60	
A.B	0.65	0.64	0.67	0.40	0.82	0.67	0.64	0.60

Table 4.6: The genetic distances, F=1-S.I (see section 3.2.3), of nine *Acorus* species calculated from OPAW-04. A.X: *A. xiangyeus*, A.L: *A. latifolius*, A.G.V: *A. gramineus* var. flavo-marginatus, A.G: *A. gramineus*, A.M: *A. macrospadiceus*, A.T: *A. tatarinowii*, A.C: *A. calamus* L., A.R: *A. rumphianus*, A.B.: *A. brevispathus*

When primer OPAW-04 was used, *A. latifolia* was distantly related to *A. gramineus*, *A. macrospadiceus* and *A. calamus*. *A. tatarinowii* and *A. rumphianus* were also distanly related. The relatedness of other species was also quite low, which ranged from 0.40 to 0.83.

The relationship is illustrated as follows:

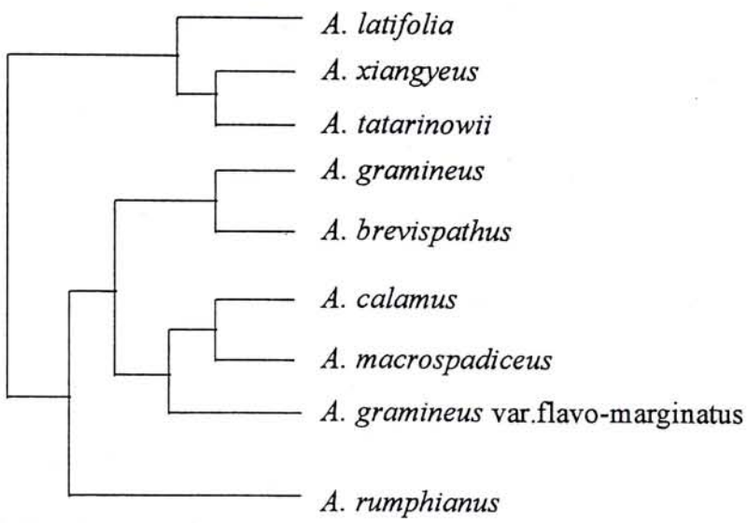

Fig.4.14: A phylogenetic tree showing the relationship among the nine *Acorus* species using primer OPAW-04.

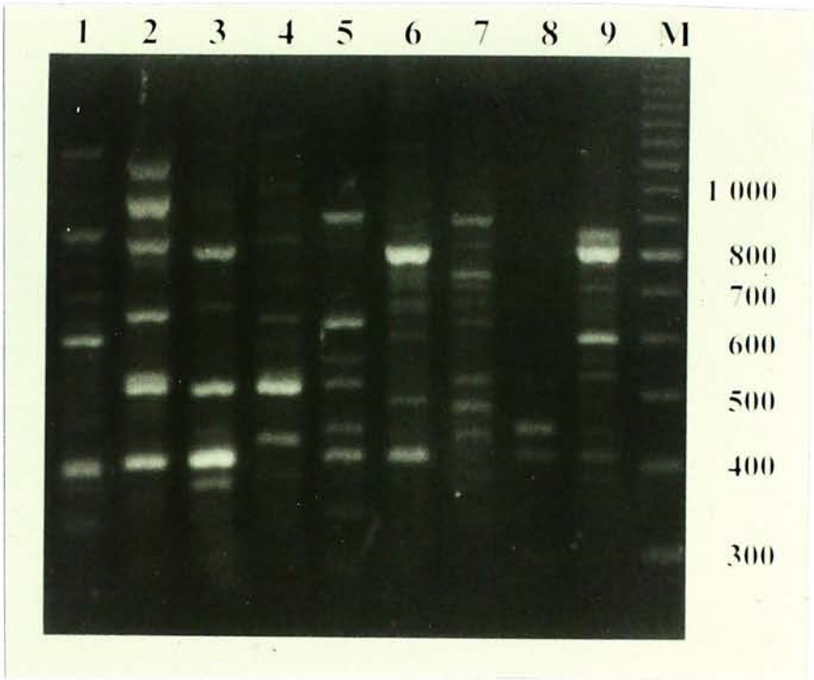

Fig.4.15: DNA fingerprint of nine *Acorus* species. It was generated by RAPD using OPAW-09: 5'ACT GGG TCG G3'. Lane 1: *A. xiangyeus*, Lane 2: *A. latifolia*, Lane 3: *A. gramineus* var. flavo-marginatus, Lane 4: *A. gramineus*, Lane 5: *A. macrospadiceus*, Lane 6: *A. tatarinowii*, Lane 7: *A. calamus* L., Lane 8: *A. rumphianus*, Lane 9: *A. brevispathus*. M: 100 bp ladder marker.

The bands ranged from 300 to 1 200 bp. This primer was good for identification as the strong bands were distinctive.

The genetic distances were tabulated as follows:

	A.X	A.L	A.G.V	A.G	A.M	A.T	A.C	A.R
A.L	0.56							
A.G.V	**1.00**	0.75						
A.G	**1.00**	**1.00**	0.67					
A.M	**1.00**	0.82	0.45	0.56				
A.T	0.64	0.80	0.60	0.75	0.54			
A.C	0.78	0.75	0.75	0.67	0.82	0.80		
A.R	0.75	0.43	0.71	**1.00**	0.80	0.78	0.71	
A.B	**1.00**	0.71	0.43	0.60	0.40	0.33	0.71	0.67

Table 4.7: The genetic distances, F=1-S.I (see section 3.2.3), of nine *Acorus* species calculated from OPAW-09. A.X: *A. xiangyeus*, A.L: *A. latifolius*, A.G.V: *A. gramineus* var. flavo-marginatus, A.G: *A. gramineus*, A.M: *A. macrospadiceus*, A.T: *A. tatarinowii*, A.C: *A. calamus* L., A.R: *A. rumphianus*, A.B.: *A. brevispathus*

Using primer OPAW-09, six pairs of species (highlighted) were distantly related.. The genetic distances of the majority species were above 0.60. It implied that their relatedness was relatively low.

The relationship is illustrated as follows:

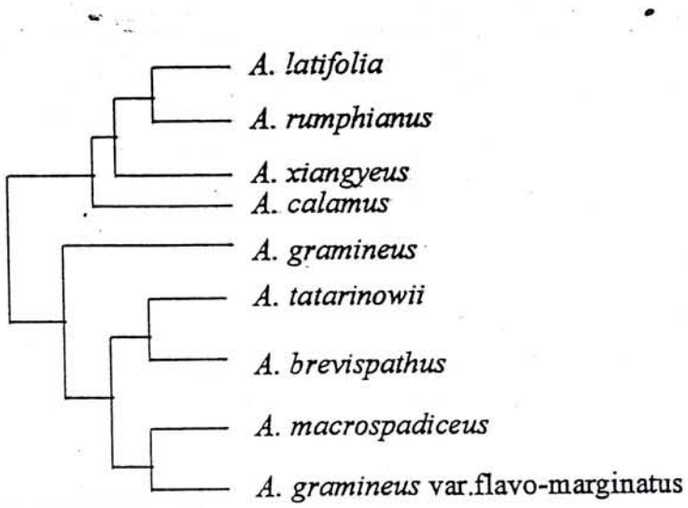

Fig.4.16: A phylogenetic tree showing the relationship among the nine *Acorus* species using OPAW-09.

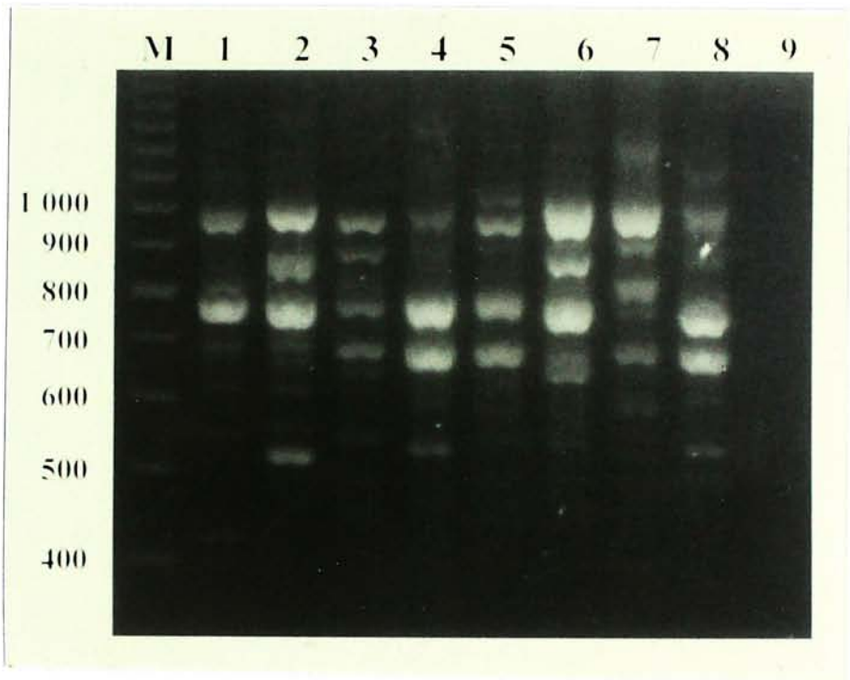

Fig.4.17: DNA fingerprint of nine *Acorus* species. It was generated by RAPD using OPC-19: 5'GTT GCC AGC C3'. Lane 1: *A. xiangyeus*, Lane 2: *A. latifolia*, Lane 3: *A. gramineus* var. flavo-marginatus, Lane 4: *A. gramineus*, Lane 5: *A. macrospadiceus*, Lane 6: *A. tatarinowii*, Lane 7: *A. calamus* L., Lane 8: *A. rumphianus*, Lane 9: *A. brevispathus*. M: 100 bp ladder marker.

The bands produced were in the range of 400 to 1 000 bp. Three common bands of about 700, 750 and 920 bp were observed among all species. Most of the other bands were of weak intensity.

The genetic distances are tabulated as follows:

	A.X	A.L	A.G.V	A.G	A.M	A.T	A.C	A.R
A.L	0.56							
A.G.V	0.33	0.20						
A.G	0.50	0.33	0.11					
A.M	0.43	0.25	0.25	0.43				
A.T	0.56	0.20	0.20	0.33	0.25			
A.C	0.43	0.25	0.25	0.43	0.00	0.25		
A.R	0.33	0.20	0.00	0.11	0.25	0.20	0.25	
A.B	1.00	0.67	0.67	0.60	0.50	0.67	0.50	0.67

Table 4.8: The genetic distances, F=1-S.I (see section 3.2.3), of nine *Acorus* species calculated from OPC-19. A.X: *A. xiangyeus*, A.L: *A. latifolius*, A.G.V: *A. gramineus* var. flavo-marginatus, A.G: *A. gramineus*, A.M: *A. macrospadiceus*, A.T: *A. tatarinowii*, A.C: *A. calamus* L., A.R: *A. rumphianus*, A.B.: *A. brevispathus*

Using primer OPC-19, *A. xiangyeus* and *A. brevispathus* were distantly related while the pairs of *A. gramineus* var. flavo-marginatus, *A. rumphianus* and *A. macrospadiceus*, *A. calamus* were identical in the DNA fingerprint. The relatedness of other species were quite high except *A. brevispathus* which had the genetic distances above 0.50.

The relationship is illustrated as follows:

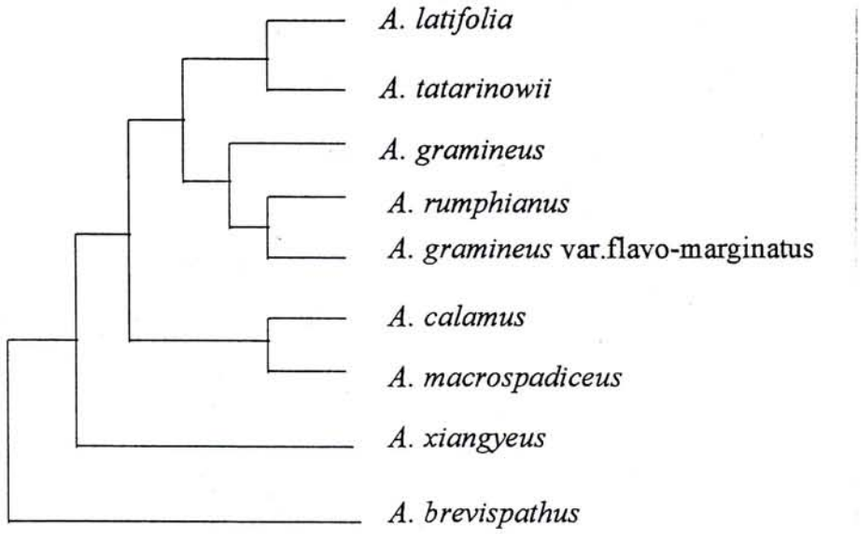

Fig.4.18: A phylogenetic tree showing the relationship among the nine *Acorus* species using primer OPC-19.

A phylogenetic tree of individual primer is plotted (Figs.4.12 to 4.18). The grouping of the nine species were slightly different. For instances, *A. brevispathus and A. macrospadiceus* were classified into one group using primers Seq2 (Fig.4.10) and OPAA-06 (Fig.4.12). However, when other primers were used, they were not the most related species. Different primers may amplify different loci that explain the variation in the fingerprints and hence the phylogeny. Therefore, a consensus tree represented the resultant relationship of various species is plotted. (Fig.4.19). It is noticed that *A. latifolia, A. tatarinowii* and *A. xiangyeus* were closely related and formed a cluster in the consensus tree as well as in the individual phylogenetic trees.

Apart from that, *A. tatarinowii* and *A. gramineus* were placed in two clusters. Some botanists claimed that *A. tatawinowii* was just another form of *A. gramineus* and could not be classified as an individual species. But from the fingerprints, these two samples were actually having different genetic makeup. They might be classified as two species.

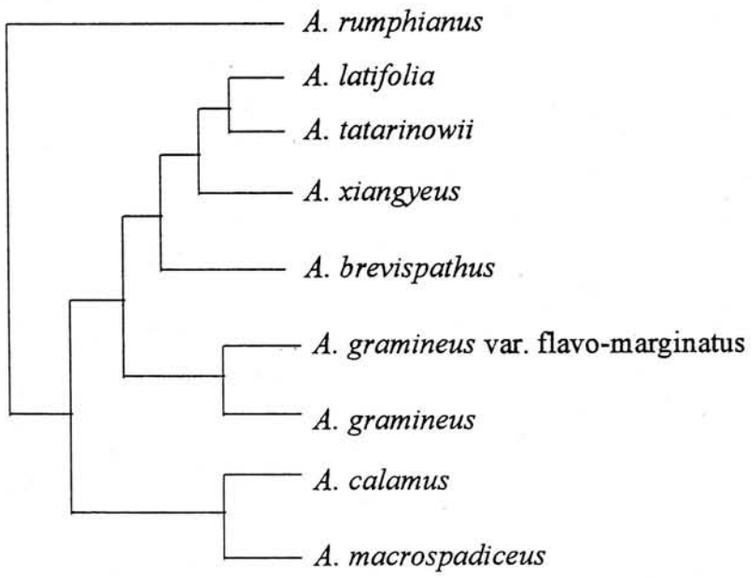

Fig.4.19: A consensus tree showing the relationship between the nine *Acorus* species. It was constructed by summing up individual phylogenetic trees from each primer in the computer program PHYLIP.

Another point we should notice is the relationship between *A. tatawinowii* and *A. calamus* L. In the past, people sometimes misused *A. calamus* L. as *A. tatawinowii* because they look alike but the pharmacological action of *A.tatawinoii* is much stronger than *A.calamus* L. Thus the misuse of the two species would affect the pharmacological efficacy. From the consensus tree, both species were not in the same cluster and they had different fingerprints. It is easier to distinguish them by the random-primed polymerase chain reactions.

Chapter 5 Studies of *Epimedium* by Random-Primed PCRs

Chapter 5 Studies of *Epimedium* by Random-Primed PCRs

5.1 Introduction

Epimedium < Yinyanghuo > (Fig.5.1) belongs to the family Berberidaceae. It is distributed in the West and Northwest of China. There are about twenty *Epimedium* species around the world. Thirteen species are found in China, two of them are variants (Ying, 1975). The aerial part of this herb is used for tonifying kidneys, impotence and atrophy. Owing to the similar morphological and anatomical characters, the sources of *Epimedium* sold on the market sometimes cannot be certain. Consequently, the quality of the herbs is greatly affected.

Fig.5.1: The morphology of *Epimedium* species. There are about twenty *Epimedium* species around the world. New species are still being identified.

Morphological and chemotaxonomical classifications of *Epimedium* species are obscure because it is known that hybridization between species occurs. It results in the diversification of populations (Takemoto *et al*, 1975). We have tried to study six *Epimedium* species by random-primed PCRs which forms the basis for in depth identification.

5.2 Materials and Methods

5.2.1. Plant Materials. Six *Epimedium* species were collected from China and identified by Professor D. W. Shi.

5.2.2. DNA Extraction and Random-Primed PCRs. DNA extraction is described in Section 2.2. AP-PCR and RAPD were carried out in 25 ul containing the same reagents mentioned in Section 2.6, except 10 ng DNA was used in both AP-PCR and RAPD. Conditions were same as that in Section 2.6 but a step of incubation at 95^0C for five minutes was performed before the actual reaction cycles. There was a final extension at 72^0C for 10 minutes.

5.3 Results and Discussion

5.3.1 DNA Extraction

The DNA extracted was of high molecular weight although part of them were degraded (Fig.5.2).

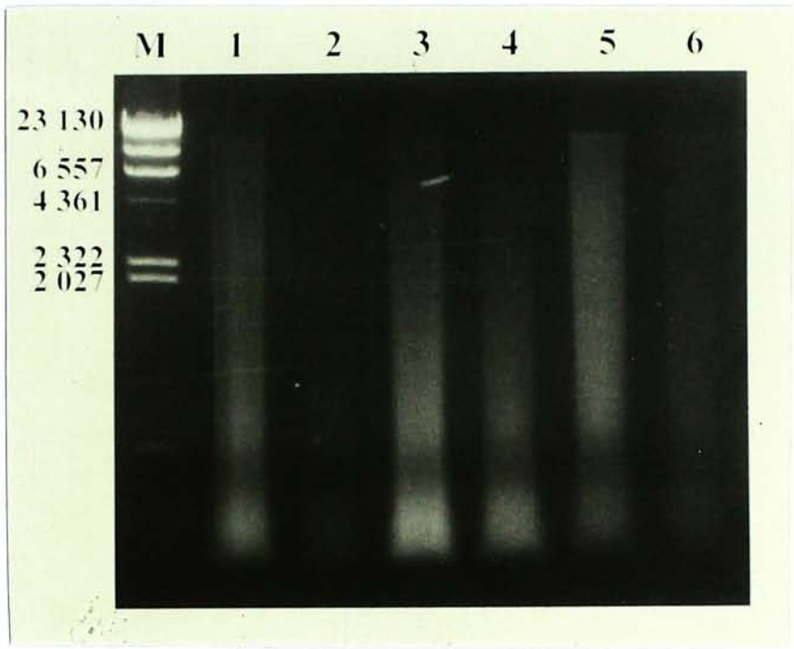

Fig.5.2: The DNA isloated from the six *Epimedium* species. Lane 1: *E. brevicornum*, Lane 2: *E. saggitatum*, Lane 3: *E. wushanense*, Lane 4: *E. koreanum*, Lane 5: *E. pubescens*, Lane 6: *E. leptorrhizum*. M: λ*Hin*dIII digested marker.

5.3.2 DNA Fingerprinting

Fifty-seven primers (Table 4.1) were tested on the *Epimedium* species. Eight of them gave informative fingerprints. They are shown in Figs.5.3 to 5.10. Table 5.2 indicates the genetic distance matrix between individual species calculated according to the Nei and Li's method (Section 3.2.3).

RAPD Primers	AP-PCR Primers
OPA-01 to 04	Gal K
OPB-01, 06, 07 and 10	M13 forward
OPC-08 and 20	M13 backward
OPD-02 and 03	Seq 2
OPAA-01 to 04, 11 to 15 and 17	TCS forward
OPAM-02, 06, 12, 14 to 16	TCS backward
OPAW-01 to 15	primers #14 to 20

Table 5.1: The list of primers tested on the *Epimedium* species. The sequences of the primers are listed in Appendix II.

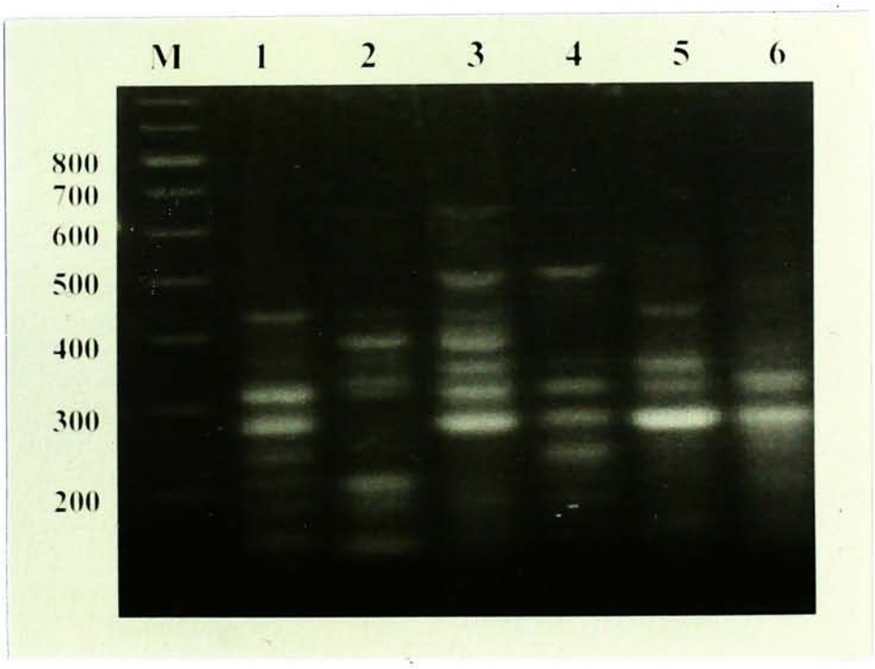

Fig.5.3: DNA fingerprints of six *Epimedium* species. Lane 1: *E. brevicornum*, Lane 2: *E. saggitatum*, Lane 3: *E. wushanense*, Lane 4: *E. koreanum*, Lane 5: *E. pubescens*, Lane 6: *E. leptorrhizum*. M: 100 bp ladder marker. Fingerprints were generated by AP-PCR using M13F: 5'CGC CAG GGT TTT CCC AGT CAC GAC3'.

The bands produced ranged from 100 to 700 bp. Two bands of around 300 bp were shared among most of the six species. The majority of bands generated were distinctive and of high intensity.

The genetic distances are tabulated as follows:

	E.B	E.S	E.W	E.K	E.P
E.S	0.33				
E.W	0.50	0.57			
E.K	0.45	0.54	0.54		
E.P	0.50	0.80	0.40	0.78	
E.L	0.43	0.78	0.56	0.50	0.60

Table 5.2. The genetic distance, F=1-S.I. (see section 3.2.3), of *Epimedium* species calculated from primer M13 forward. *E. brevicornum*, E.S: *E. saggitatum*, E.W: *E. wushanense*, E.K: *E. koreanum*, E.P: *E. pubescens*, E.L: *E.leptorrhizum*.

Using primer M13 forward, the genetic distances ranged from 0.33 to 0.80.

The relationship is illustrated as follows:

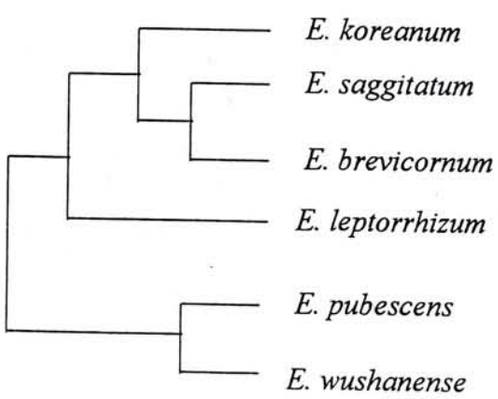

Fig.5.4: A phylogenetic tree showing the relationship among the six *Epimedium* species using M13 forward primer.

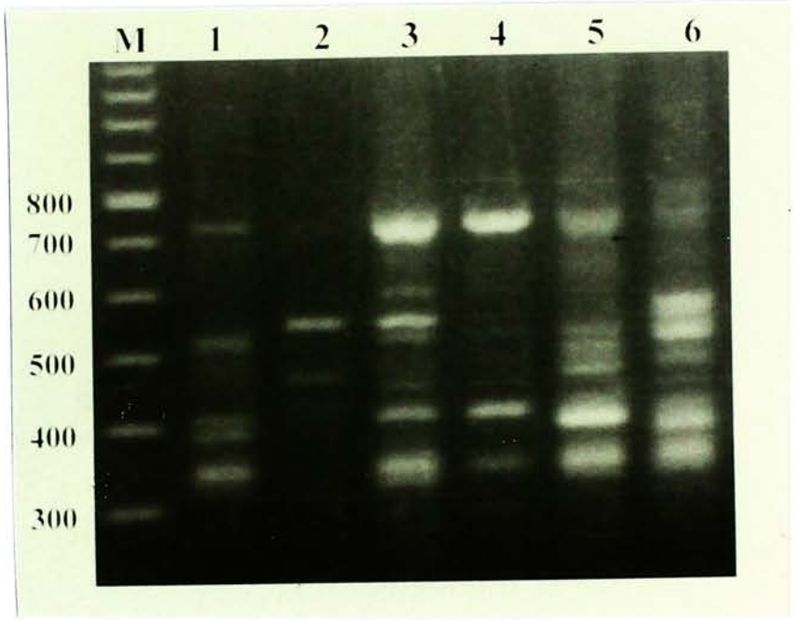

Fig.5.5: DNA fingerprints of six *Epimedium* species. Lane 1: *E. brevicornum*, Lane 2: *E. saggitatum*, Lane 3: *E. wushanense*, Lane 4: *E. koreanum*, Lane 5: *E. pubescens*, Lane 6: *E. leptorrhizum*. M: 100 bp ladder marker. Fingerprints were generated by AP-PCR using primer#18: 5'CAT CGG ATC CAC CAC GTC3'.

The bands produced ranged from 300 to 700 bp. A band of 320 bp was shared among most of the species except *E. saggitatum* which had only two bands in this fingerprint. Dominant bands were observed in the six species.

The genetic distances are tabulated as follows:

	E.S	E.W	E.K	E.P
E.W	0.11			
E.K	0.25	0.11		
E.P	0.45	0.33	0.27	
E.L	0.33	0.20	0.11	0.17

Table 5.3. The genetic distance, F=1-S.I. (see section 3.2.3), of *Epimedium* species calculated from primer#18. E.S: *E. saggitatum*, E.W: *E. wushanense*, E.K: *E. koreanum*, E.P: *E. pubescens*, E.L: *E.leptorrhizum*.

No band was produced in *E. brevicornum* using primer#18 in other repeated reactions, so it was excluded in the calculation. The relatedness of the five species was relatively close as their genetic distances were ranged from 0.11 to 0.45.

The relationship is illustrated as follows:

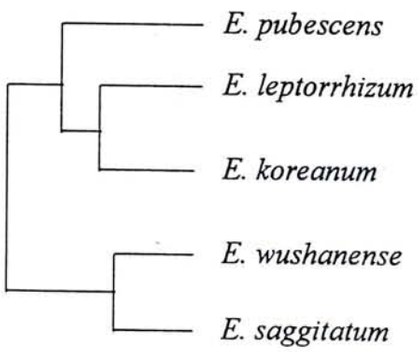

Fig.5.6: A phylogenetic tree showing the relationship among the five *Epimedium* species using primer#18.

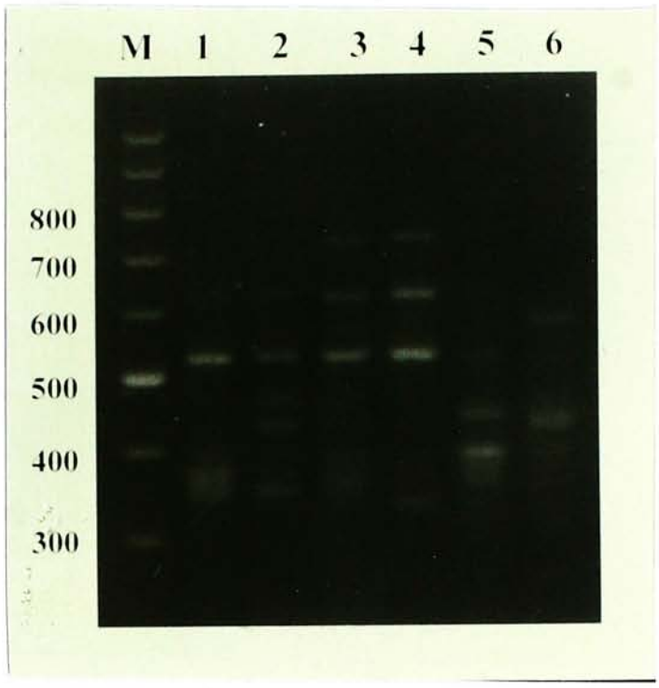

Fig.5.7: DNA fingerprints of six *Epimedium* species. Lane 1: *E. brevicornum*, Lane 2: *E. saggitatum*, Lane 3: *E. wushanense*, Lane 4: *E. koreanum*, Lane 5: *E. pubescens*, Lane 6: *E. leptorrhizum*. M: 100 bp ladder marker. Fingerprints were generated by AP-PCR using primer#20: 5'GCC CCC TTT ATC AAC GAT TCT3'.

The bands ranged from 300 to 800 bp. Some vague bands around 400 bp were present. They were not included in the identification and phylogenetic studies.

The genetic distances are tabulated as follows:

	E.B	E.S	E.W	E.K	E.P
E.S	0.39				
E.W	0.40	0.39			
E.K	0.41	0.64	0.37		
E.P	0.45	0.47	0.39	0.58	
E.L	0.38	0.46	0.76	0.39	0.33

Table 5.4. The genetic distance, F=1-S.I. (see section 3.2.3), of *Epimedium* species calculated from primer#20. E.B: *E.brevicornum*, E.S: *E. saggitatum*, E.W: *E. wushanense*, E.K: *E. koreanum*, E.P: *E. pubescens*, E.L: *E.leptorrhizum*.

Using primer#20, *E. wushanense* was distantly related to *E. leptorrhizum* while the latter was more closely related to *E. pusbescens.*

The relationship is illustrated as follows:

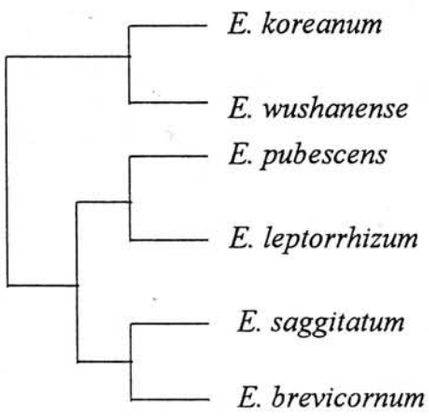

Fig.5.8: A phylogenetic tree showing the relationship among the six *Epimedium* species using primer#20.

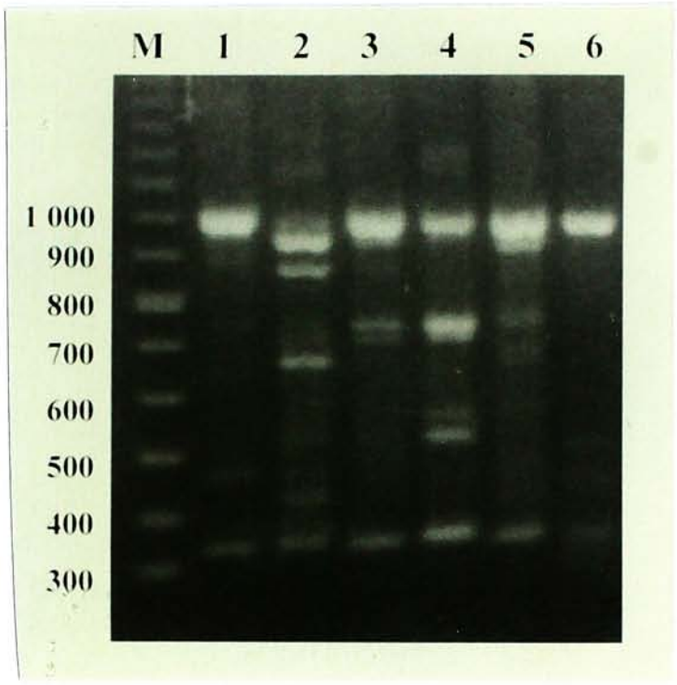

Fig.5.9: DNA fingerprints of six *Epimedium* species. Lane 1: *E. brevicornum*, Lane 2: *E. saggitatum*, Lane 3: *E. wushanense*, Lane 4: *E. koreanum*, Lane 5: *E. pubescens*, Lane 6: *E. leptorrhizum*. M: 100 bp ladder marker. Fingerprints were generated by RAPD using OPAA-01: 5'TGG ACC GGT G3.

The bands produced were in the range of 300 to 1 000 bp. Two distinctive bands of about 300 and 950 bp were present in most of the species. No strong and obvious polymorphic bands was found in. *E. brevicorum*, *E.pubescens* and *E. leptorrhizum* for identification.

The genetic distances are tabulated as follows:

	E.B	E.S	E.W	E.K	E.P
E.S	0.60				
E.W	0.45	0.38			
E.K	0.33	0.64	0.17		
E.P	0.50	0.60	0.27	0.33	
E.L	0.67	1.00	0.56	0.43	0.33

Table 5.5. The genetic distance, F=1-S.I. (see section 3.2.3), calculated from primers OPAA-01 of *Epimedium* species. E.B: *E.brevicornum*, E.S: *E. saggitatum*, E.W: *E. wushanense*, E.K: *E. koreanum*, E.P: *E. pubescens*, E.L: *E.leptorrhizum*.

Using primer OPAA-01, *E. saggitatum* was distantly related to *E. leptorrhizum*. *E.wushanense* was closely related to *E. koreanum*.

The relationship is illustrated as follows:

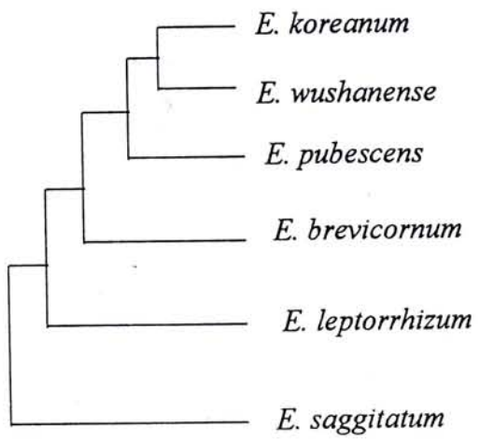

Fig.5.10: A phylogenetic tree showing the relationship among the six *Epimedium* species using primer OPAA-01.

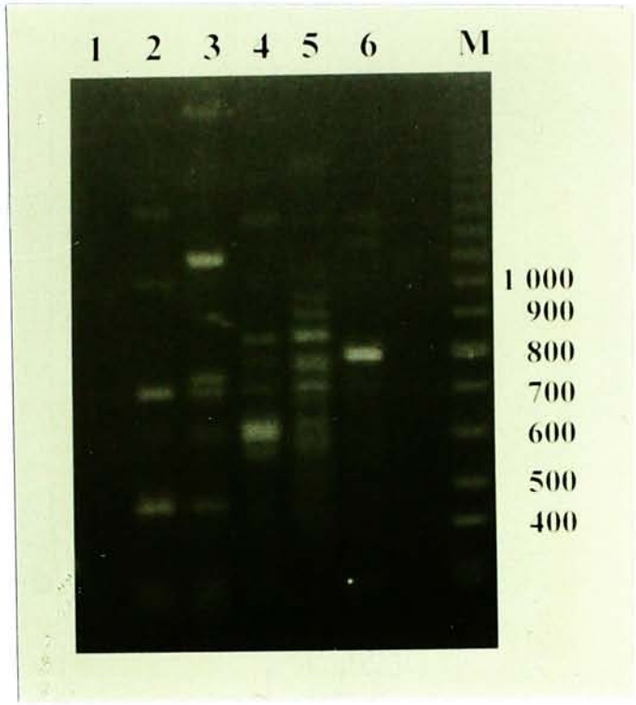

Fig.5.11: DNA fingerprints of six *Epimedium* species. Lane 1: *E. brevicornum*, Lane 2: *E. saggitatum*, Lane 3: *E. wushanense*, Lane 4: *E.koreanum*, Lane 5: *E. pubescens*, Lane 6: *E. leptorrhizum*. M: 100 bp ladder marker. Fingerprints were generated by RAPD using OPAA-17: 5'GAG CCC GAC T3'.

The bands were in the range of 400 to 1 300 bp. No band was produced in *E. brevicornum* in this reaction. Bands were mainly concentrated between 600 to 900 bp. Some of them were shared between individual species. Other scattered bands were unique to each species, for example, a prominent band of 1 100 bp was found in *E. wushanense*.

The genetic distances are tabulated as follows:

	E.B	E.S	E.W	E.K	E.P
E.S	0.67				
E.W	0.75	0.60			
E.K	0.67	0.75	0.80		
E.P	0.80	0.67	0.86	0.67	
E.L	0.33	0.60	0.71	0.60	0.78

Table 5.6. The genetic distance, F=1-S.I. (see section 3.2.3), of *Epimedium* species calculated from primer OPAA-17. E.B: *E.brevicornum*, E.S: *E. saggitatum*, E.W: *E. wushanense*, E.K: *E. koreanum*, E.P: *E. pubescens*, E.L: *E.leptorrhizum*.

Using primer OPAA-17, the relatedness of the six *Epimedium* species was relatively low. The genetic distances of the majority species were above 0.6.

The relationship is illustrated as follows:

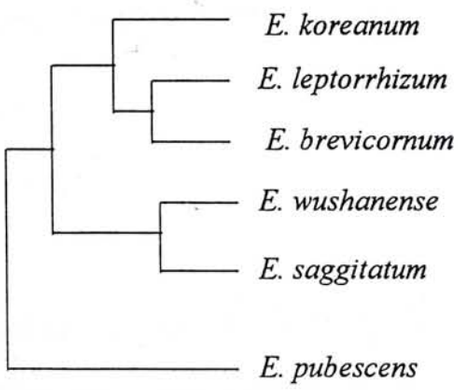

Fig.5.12: A phylogenetic tree showing the relationship among the six *Epimedium* species using primer OPAA-17.

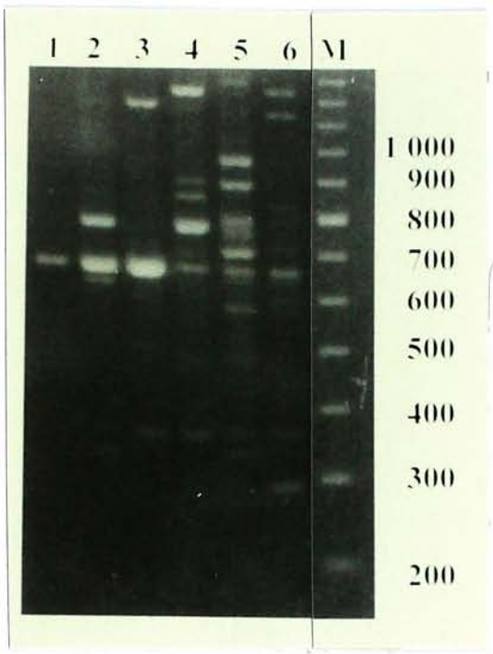

Fig.5.13: DNA fingerprints of six *Epimedium* species. Lane 1: *E. brevicornum*, Lane 2: *E. saggitatum*, Lane 3: *E. wushanense*, Lane 4: *E.koreanum*, Lane 5: *E. pubescens*, Lane 6: *E. leptorrhizum*. M: 100 bp ladder marker. Fingerprints were generated by RAPD using OPB-01: 5'GTT TCG CTC C3'.

The bands produced ranged from 300 to 1 300 bp. The majority of the bands were between 600 to 900 bp. They were quite distinct although some bands in *E. leptorrhizum* were weak which are excluded in the scoring process. A prominent common band of 650 bp was found in all the six species.

The genetic distances are tabulated as follows:

	E.B	E.S	E.W	E.K	E.P
E.S	0.60				
E.W	0.50	0.71			
E.K	0.71	0.60	0.56		
E.P	0.71	0.60	0.78	0.67	
E.L	0.67	0.78	0.56	0.45	0.82

Table 5.7. The genetic distance, F=1-S.I. (see section 3.2.3), of *Epimedium* species calculated from primer OPB-01. E.B: *E.brevicornum*, E.S: *E. saggitatum*, E.W: *E. wushanense*, E.K: *E. koreanum*, E.P: *E. pubescens*, E.L: *E.leptorrhizum.*

Using primer OPB-01, the genetic distances of the majority species were above 0.6. It suggested that they were not closely related.

The relationship is illustrated as follows:

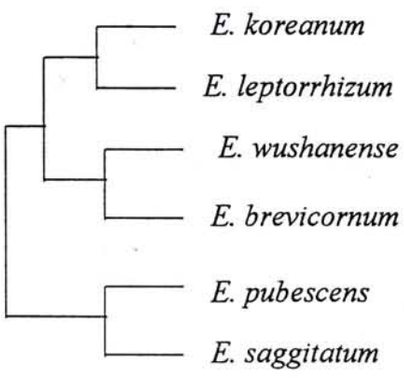

Fig.5.14: A phylogenetic tree showing the relationship among the six *Epimedium* species using primer OPB-01.

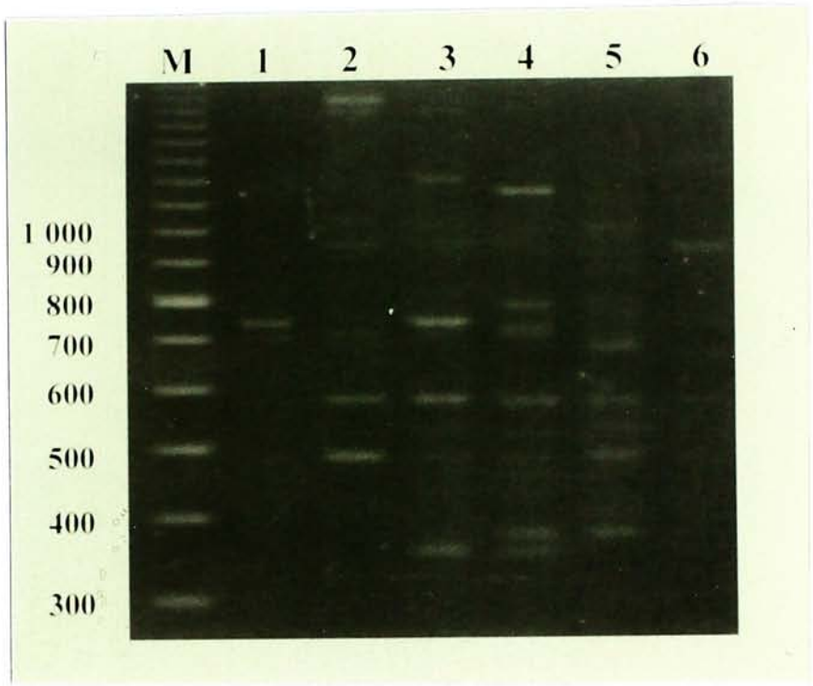

Fig.5.15: DNA fingerprints of six *Epimedium* species. Lane 1: *E. brevicornum*, Lane 2: *E. saggitatum*, Lane 3: *E. wushanense*, Lane 4: *E.koreanum*, Lane 5: *E. pubescens*, Lane 6: *E. leptorrhizum*. M: 100 bp ladder marker. Fingerprints were generated by RAPD using OPB-06: 5'TGC TCT GCC C3'.

The bands produced were in the range of 300 to 1 200 bp. One to six prominent bands were detected among the six species; others were weak and not distinct. A 580 bp prominent band was found in *E. saggitatum, E. wushanense, E.koreanum* and *E. pubescens*.

The genetic distances are tabulated as follows:

	E.B	E.S	E.W	E.K	E.P
E.S	0.56				
E.W	0.56	0.50			
E.K	0.67	0.47	0.60		
E.P	0.83	0.60	0.60	0.56	
E.L	0.43	0.40	0.60	0.69	0.54

Table 5.8. The genetic distance, F=1-S.I. (see section 3.2.3), of *Epimedium* species calculated primer OPB-06.. E.B: *E.brevicornum*, E.S: *E. saggitatum*, E.W: *E. wushanense*, E.K: *E. koreanum*, E.P: *E. pubescens*, E.L: *E.leptorrhizum.*.

Using OPB-06, the genetic distances of the majority species were around 0.6. *E. brevicornum* was distantly related to *E. pubescens*.

The relationship is illustrated as follows:

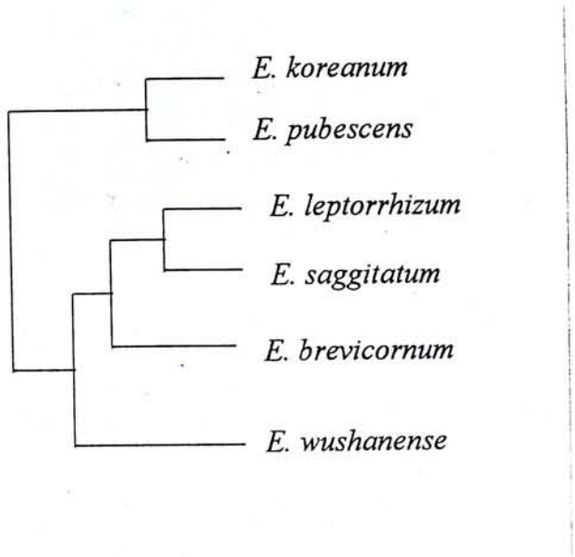

Fig.5.16: A phylogenetic tree showing the relationship among the six *Epimedium* species using primer OPB-06.

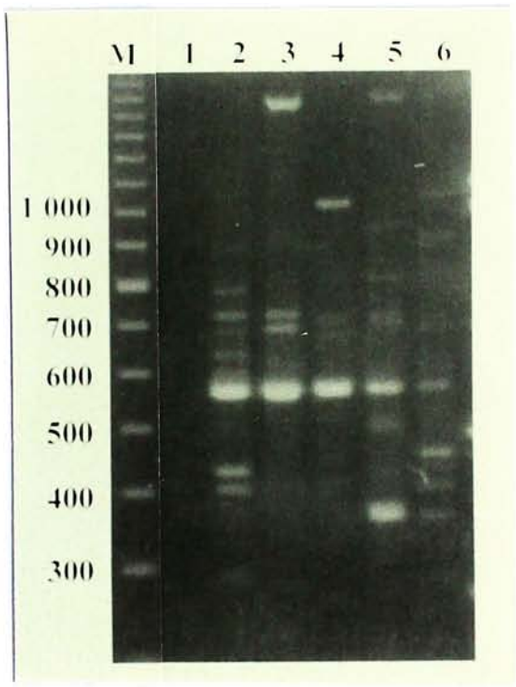

Fig.5.17: DNA fingerprints of six *Epimedium* species. Lane 1: *E. brevicornum*, Lane 2: *E. saggitatum*, Lane 3: *E. wushanense*, Lane 4: *E.koreanum*, Lane 5: *E. pubescens*, Lane 6: *E. leptorrhizum*. M: 100 bp ladder marker. Fingerprints were generated by RAPD using OPAW-09: 5'ACT GGG TCG G3'.

The bands ranged from 300 to 1 400 bp. No band was produced in *E. brevicornum* using this primer. A distinctive band of 550 bp was shared among five species. Other bands were shared between individual species.

The genetic distances are tabulated as follows:

	E.S	E.W	E.K	E.P
E.W	0.64			
E.K	0.64	0.33		
E.P	0.69	0.25	0.50	
E.L	0.43	0.78	0.56	0.64

Table 5.9. The genetic distance, F=1-S.I. (see section 3.2.3), of *Epimedium* species calculated from primer OPAW-09.. E.B: *E.brevicornum*, E.S: *E. saggitatum*, E.W: *E. wushanense*, E.K: *E. koreanum*, E.P: *E. pubescens*, E.L: *E.leptorrhizum*.

No band was produced in *E. brevicornum* using primer OPAW-09. Thus it was excluded in the genetic distance calculation. The genetic distances ranged from 0.25 to 0.78. *E. wushanense* was closely related to *E. pubescens*.

The relationship is illustrated as follows:

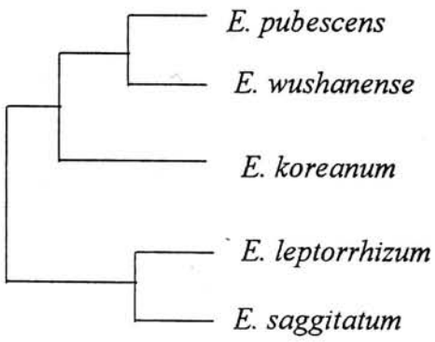

Fig.5.18: A phylogenetic tree showing the relationship among the five *Epimedium* species using primer OPAW-09.

In the past, some species of *Epimedium* were identified as *E. saggitatum* (Xu, 1994). It was due to the lack of an effective identification method. Morphological characteristics of leaf, stem and root are similar and unable to distinguish different species. Chemical analysis on the active component icariin was also difficult to identify them. It was because the quantity of the chemical was affected by environment and harvesting period. The bands produced in the six species ranged from 100 to 1 300 bp among the fingerprints of the informative primers. Each species had their own banding pattern. It suggested that they were of different genetic composition.

A consensus tree (Fig.5.19) was plotted by summing up individual phylogenetic trees from each primers. It showed that *E. saggitatum* and *E. koreanum* were grouped into two clusters. It was in accordance with Nakai (1996) who reported that these two species formed two clusters using the same cluster analysis.

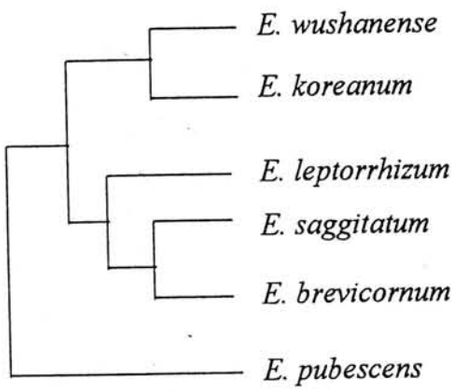

Fig.5.19: A consensus tree showing the relationship between the six *Epimedium* species.

Chapter 6 Application of AP-PCR in Commercial Ginseng Products

Chapter 6 Application of AP-PCR in Commercial Ginseng Products

6.1 Introduction

Since ginseng is regarded as a superior herb in the Chinese medicine, many people like to take it for therapy or as a health food. In order to satisfy the demand, different ginseng products in the form of tea bags, powder or pills (Fig.6.1) are available on the market. Quality control is very important as dishonest merchant would disguise other adulterants as genuine ginsengs.

Fig.6.1: A variety of commercial ginseng products are available on the market.

In this section, ten ginseng products available on the markets were collected. Their compositions were tested using the random-primed PCRs. The aim of this section is to find if fingerprints similar to those for ginseng roots are generated, so as to provide an additional tool in the quality control of the commercial ginseng products.

6.2 Materials and Methods

6.2.1 Materials

Ten ginseng products were supplied by the CMMRC. Their sources are summarized in Table 6.1.

Sample no	Code no	Characteristics of products
1	148-3	granular root
2	149-4	powder of water extract
3	-	pills containing powder
4	96007	powder
5	96008	powder
6	96009	powder
7	96010	powder
8	96011	powder
9	168	root
10	169	powder from fresh root

Table 6.1: Ten commercial ginseng products and their characteristics.

6.2.2 DNA Extraction and Random-Primed PCRs

They were performed according to Sections 2.2. and 2.6. The reaction volume in AP-PCR was 25 ul.

6.2.3 Data Analysis

The DNA profile of the samples were compared with the reference American and Oriental ginseng for the existence of general patterns and any specific band for a given

sample. The detection of all American or Oriental ginseng bands indicated the presence of American and Oriental ginseng in the samples respectively. Existence of characteristic bands of both American and Oriental ginsengs were concluded as a mixture of them. No characteristic band of the reference ginsengs found was concluded as absence of either ginsengs.

6.3 Results and Discussion

6.3.1 DNA Isolation

Fig 6.2 shows the DNA extracted from the commercial ginseng products. High molecular weight DNA were extracted from samples #1, #3 to #9, although the DNA was degraded to different extent and RNA was present in sample #5. No detectable DNA was extracted from samples #2 and #10. A lot of RNA was seen in sample #10 on the agarose gel.

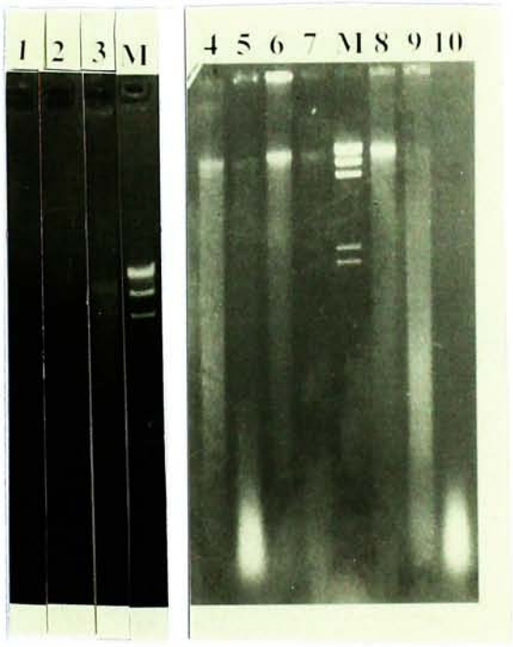

Fig.6.2: The DNA extracted from the ten commercial ginseng products. Lanes 1 to10 are the samples #1 to #10. 10μl of DNA solution was loaded onto 1% agarose gel. M: λ*Hin*dIII digested marker.

Further purification of DNA was achieved by CsCl gradient ultracentrifugation and ethanol precipitation. The results were the same as before. It may be attributed that the commercial products were processed into powder or even fried with sugar. For instances, sample #2 was water soluble ginseng tea. Some of the DNA might have already degraded during processing.

6.3.2 AP-PCR Analysis

Fifteen primers (Table 6.2) were tested on samples #1 and #2. Only AP-PCR primers produced bands sufficiently good for differentiation. Thus all samples were analysed by AP-PCR. Altogether nine AP-PCR primers were tested and five of them could identify most of the samples. Each reaction had been repeated at least twice. The results are summarized in Table 6.3. and the fingerprints are shown in Figs 6.3 to 6.5.

RAPD Primers	AP-PCR Primers
OPC-04, 05, 19 & 20	Gal K
OPAA-17	M13 forward
OPAW-04 & 09	M13 backward
	Seq2
	TCS backward
	primer#10
	primer#13
	primer#18
	primer#20

Table 6.2: The list of primers tested on the commercial products. Sequences of the primers are listed in Appendix II.

	#13	#18	#20	TCSb	Gal K	Conclusion	average %confidence
Sample 1	**AG/G** (50%)	**AG** (100%)	- (100%)	**AG** (33.3%)	- (50%)	**AG**	66.7
Sample 2	**G** (25%)	**G** (33.3%)	- (50%)	**AG** (12%)	- (66.7%)	-	58.4
Sample 3	**AG** (100%)	**AG** (75%)	**AG** (100%)	**AG** (100%)	- (66.7%)	**AG**	93.7
Sample 4	**G** (66.7%)	**AG** (73%)	**G** (50%)	**G** (66.7%)	**G** (50%)	**G**	58.3
Sample 5	**AG/G**(66.7%)	**G** (40%)	**G** (100%)	**G** (50%)	**G** (100%)	**G**	72.5
Sample 6	**AG** (100%)	**AG** (40%)	**AG** (100%)	**AG** (100%)	**G** (100%)	**AG**	85
Sample 7	- (100%)	**AG** (28%)	- (100%)	**AG/G**(50%)	- (100%)	-	100
Sample 8	**AG** (100%)	**AG** (100%)	**AG** (100%)	**AG** (100%)	**AG** (100%)	**AG**	100
Sample 9	**AG** (75%)	**AG** (100%)	**AG** (100%)	**AG** (100%)	- (100%)	**AG**	93.7
Sample 10	- (100%)	**G** (12%)	- (75%)	- (66.7%)	- (100%)	-	85.4

Table 6.3: The conclusion drawn from AP-PCR analysis on the ten ginseng samples using five primers. Primer#13:5'ATG AGC CAT GGC AGT GAG TAT CGC3', primer#18: 5'CAT CGG ATC CAC CAC GTC3', primer#20: 5'GCC CCC TTT ATC AAC GAT TCT3', TCS backward: 5'GGT GGA TCC CTA AGC ATC AAC ATT GGT3' & Gal K: 5'TAC GGT GGC GGA GCG CAG3'. AG, G indicate the sample with American or Oriental ginseng, respectively. AG/G indicates the mixture of both ginsengs. "-" means the sample does not show the patterns of AG or G. %confidence is bracketed. It is the ratio of the number of positive conclusion to the total no. of tests.

Among the samples tested, conclusions for samples #1, #3, #4, #5, #6, #8 and #9 matched with that from HPLC on ginsenosides profile. DNA fingerprints of samples #2, #7 and #10 were inconclusive (Figs.6.3 to 6.5). On the other hand, ginsenoside profile indicated that samples #2 was American ginseng while samples #7 and #10 were Oriental ginseng.

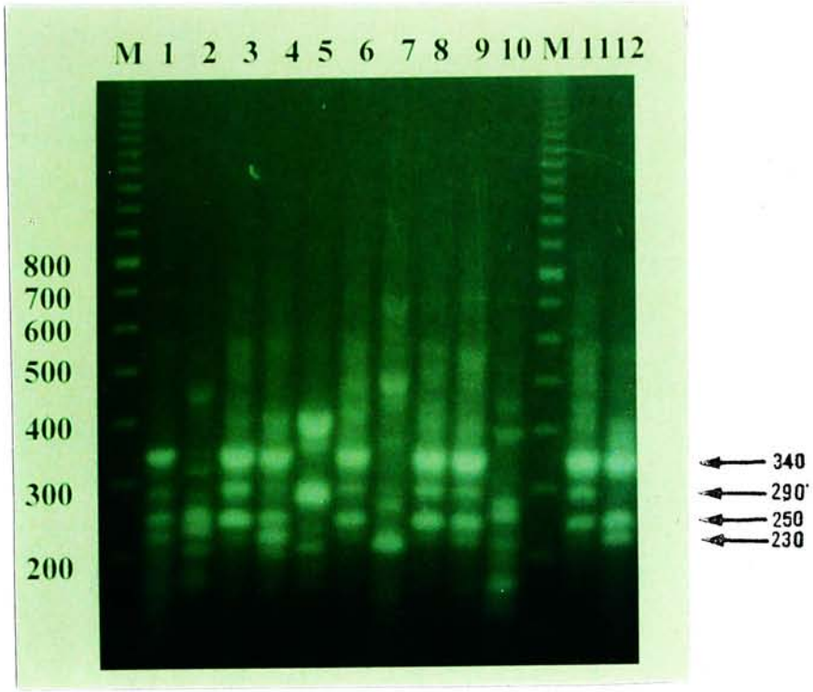

Fig 6.3: DNA fingerprint of the ten commercial ginseng products and the reference American and Oriental ginseng. It was generated using primer#13: 5'ATG AGC CAT GGC AGT GAG TAT CGC3'. Lanes 1 to 10 are Samples 1 to 10, Lane 11: American ginseng, Lane 12: Oriental ginseng. M: 100 bp ladder marker.

In Fig.6.3, the three characteristic bands (250, 290 and 340 bp) in the reference American ginseng were absent in samples #7 and #10. So no American ginseng DNA was detected. There were also three characteristic bands (230, 250 and 340 bp) in the reference Oriental ginseng, only the band of 230 bp was present in samples #7 and #10; while the other two characteristic bands were absent. Moreover, unique bands were found in samples #7 and #10. So using primer #13 could not detect any ginseng DNA in these two samples.

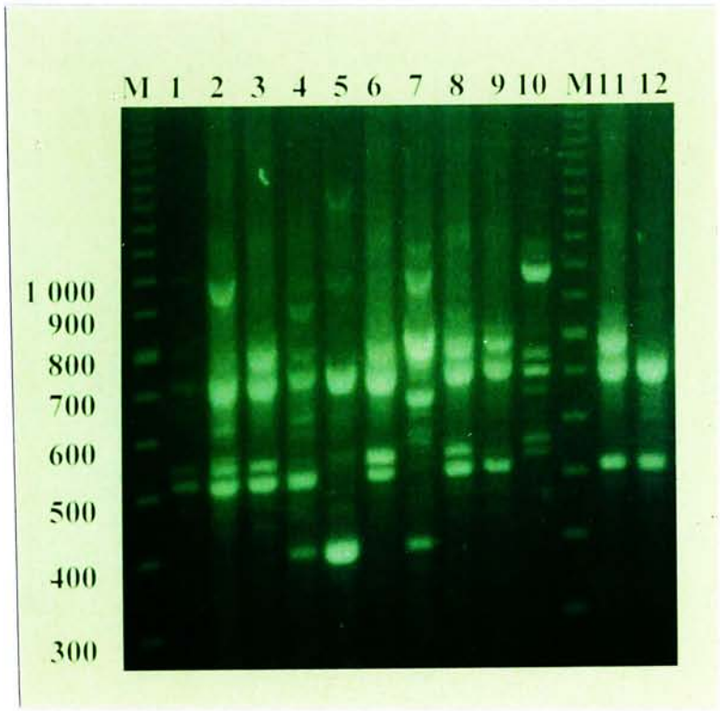

Fig 6.4: DNA fingerprint of the ten commercial ginseng products and the reference American and Oriental ginseng. It was generated using primer#18: 5'CAT CGG ATC CAC CAC GTC3'. Lanes 1 to 10 are Samples 1 to 10, Lane 11: American ginseng, Lane 12: Oriental ginseng. M: 100 bp ladder marker.

In Fig.6.4, the dominant common band of about 500 bp in both reference ginsengs were absent in samples #7 and #10. Despite having the dominant band of about 700 bp in the samples as well as the reference ginsengs, a strong band of 400 bp in sample #7 and 950 bp in sample #10 were present. Three more distinctive bands ranged from 500 to 700 bp were found in sample #10. The differences in the profile suggested that the samples might not contain either American or Oriental ginsengs DNA .

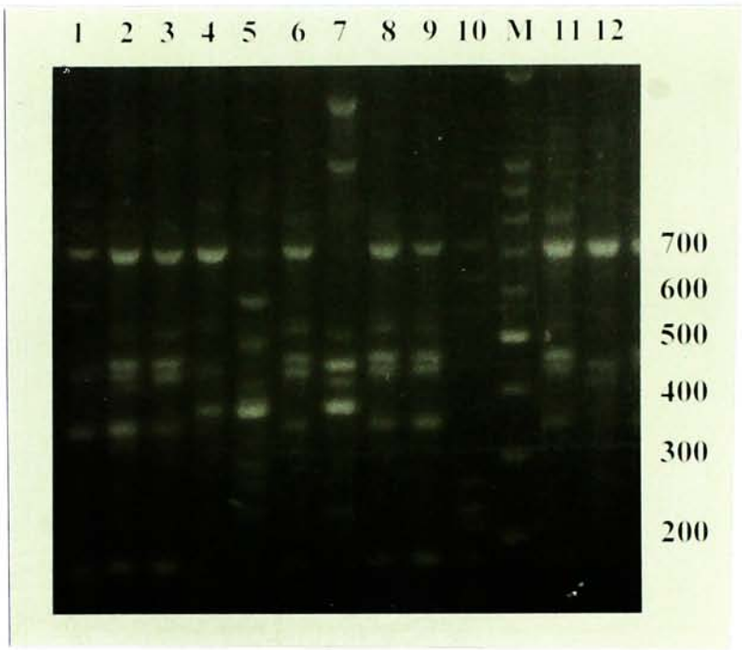

Fig 6.5: DNA fingerprint of the ten commercial ginseng products and the reference American and Oriental ginseng. It was generated using TCS backward: 5'GGT GGA TCC CTA AGC ATC AAC ATT GGT3'. Lanes 1 to 10 are Samples 1 to 10, Lane 11: American ginseng, Lane 12: Oriental ginseng. M: 100 bp ladder marker.

In Fig.6.5, only one common band of 450 bp was present between sample #7 and the reference American ginseng and no common band was found between the reference Oriental ginseng. In sample #10, only one common band of 700 bp between it and the reference American ginseng was generated.

To conclude, samples #1, #3, #6, #8 and #9 contained American ginseng. Samples #4 and #5 contained Oriental ginseng. Although no detectable DNA was extracted from samples #2 and #10. DNA fingerprints were still generated. Moreover, RNA did not affect the generation of DNA fingerprints. The DNA profile showed that these two samples were not American or Oriental ginsengs. Sample #7 had high molecular weight DNA but it gave banding patterns (Fig.6.4) which were also neither American nor Oriental ginsengs.

It was inconsistent with the results from HPLC analysed by other colleagues in the CMMRC, which detected samples #7 and #10 as Oriental ginseng. Such discrepancy might be due to the fact that the samples were processed. For instances, sample #2 was the water extract of ginseng and sugar. Samples #7 was powder from red ginseng that had been steamed. DNA property might be changed that resulted in fingerprint different from Oriental ginseng. Sample #10 was powder from fresh ginseng. HPLC analysis did not have a concrete conclusion on this sample. It showed that the sample contained substances that like Oriental ginseng. Such fingerprints might also be due to the mixture of different herbs in the samples. Further analysis on the DNA sequence of the internal transcribed spacers might provide more insight on the nature of this product.

This study also showed that processed materials had more or less affected the DNA fingerprint generation. In the samples studied, the percentage confidence on the Oriental ginseng products was lower that American ginseng products. Since the Oriental ginseng products studied in this section were from red ginseng which had been steamed, the property DNA might somehow be changed.

Chapter 7 Ribosomal DNA as a Marker in Authentication of *Panax* Species

Chapter 7 Ribosomal DNA as a Marker in Authentication of *Panax* Species

7.1 Introduction

Although the use of random-primed PCRs is effective in distinguishing different species, there is still uncertainty. The fragments of the same molecular weight in the fingerprints do not necessarily mean that they are the same. Kostia (1996) has reported two RAPD fragments of the same size in sheep and pig that do not share the same sequence; while three fragments of different sizes share a high sequence similarity. The size difference is due to varying number of microsatellites. A more precise and specific approach therefore has to be explored.

Recently, the use of ribosomal RNA genes (rDNA) as genetic marker has been successfully employed to differentiate species and strains of yeasts, parasites and soybean. (Williams *et al*, 1995; Zeller & Levy, 1995; Gu *et al*, 1994).

The ribosomal DNA of plants exist as an array of repeating units. Each unit consists of coding regions namely 18S, 5.8S and 26S. They are highly conserved that can act as an ideal primer sites. The coding regions 18S and 5.8S are separated by internal transcribed region 1 (ITS1) while 5.8S and 26S are separated by internal transcribed region 2 (ITS 2) (Hillis & Dixon, 1991) (Fig.7.1). These two regions vary considerably at the intraspecific level (Bruns *et al.*, 1991). Thus it may be used as a tool to separate the taxa into different groups. Moreover, the multiple copies of the repeat unit appear to homogenize quickly via concerted evolution (Arnheim, 1980). Consequently, it behaves like a single copy gene which allows comparison between different species.

This region can also be used for systematic studies at different taxonomic levels because the component sequences evolve at different rate (Apples & Honeycutt, 1986). The coding regions evolve slowly and are highly conserved between different species and genera (Turbeville *et al.*, 1991). The spacer regions evolve rapidly and can be used for

comparison between closely related species, subspecies or populations (Black *et al.*, 1989; Collins *et al.*, 1990; Porter & Collins, 1991).

This region is a good phylogenetic marker because it fulfils the following criteria:

i. It supplies enough consistent differences to separate the taxa into statistically supported monophyletic groups. Regions that are highly conserved will provide too few changes. Conversely, regions that are highly variable will contain too many inconsistent characters due to multiple substitutions at single positions, and alignment may be an additional problem. It would result in an inaccurate phylogeny.

ii. It evolves like a single copy region which allows comparison between species.

iii. The region has the same function in all taxa, thus it undergoes the same evolution pressure.

We have therefore attempted to reveal the phylogeny between the six *Panax* species and compare its consistence with that using the random-amplified PCRs.

7.2 Materials and Methods

7.2.1 Plant Materials

They are summarized in Table 7.1.

Sample	Origin
P. quinquefolius (P.Q)	China
P. ginseng (P.G)	China
P. notoginseng (P.N)	China
P.japonicus (P.J)	Japan
P.japonicus major (P.J.M)	China
P.trifolius (P.T)	USA
P. quinquefoilus	USA
P. quinquefoilus	Canada
P.ginseng	Russia
Mirabilis jalapa L. (M.J)	Hong Kong
Phytolacca acinosa Roxb (P.A)	Hong Kong

Table 7.1: The six *Panax* species and two adulterants in this study.

7.2.2 DNA Extraction and rDNA Amplification

DNA was extracted from six *Panax* species and two adulterants according to Section 2.2. Three *P. quinquefolius* and two *P. ginseng* collected from different regions were included to evaluate the consistence of the data. Primers 18d and 28cc were used to amplify the rDNA (Section 2.7). The amplified fragment was cut out from TAE gel and subject to Geneclean (Section 2.9.1) or it was purified directly from the PCR products using Microspin™ column (Section 2.9.2).

7.2.3. rDNA Sequencing

The rDNA of *P. quinquefolius* and *P. ginseng* (USA, Canada and Russia) were sequenced by ssDNA sequencing method (Section 2.16.3). The other six *Panax* species and the two adulterants were subcloned into TA vector and sequenced using T7 sequencing kit from Pharmacia and USB Sequenase kit from Amersham, respectively. The sequences of the six *Panax* samples were confirmed by direct PCR sequencing using SequiTherm Cycle sequencing kit. Primers were further designed for complete sequencing in both strands of DNA. The locations and sequences of different primers are shown in Fig.7.1.

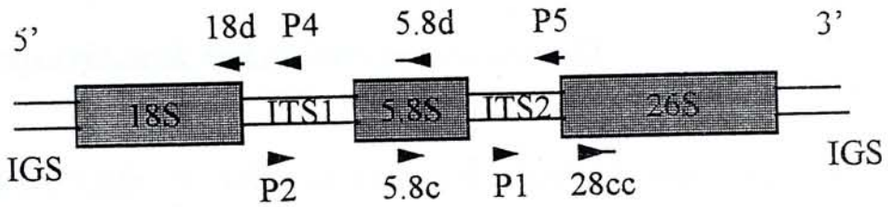

Fig.7.1: The gene-spacer-gene arrangement of plant ribosomal RNA genes. The 3 subunits: 18S, 5.8S and 26S are separated by internal transcribed spacers (ITS 1 and 2). The repeat unit is separated by intergenic spacer (IGS). The sizes of ITS1 & ITS2 in *Panax* species are about 242bp and 224bp respectively. A number of primers are used for the study of *Panax* rDNA. 18d:5'CAC ACC GCC CGT CGC TCC TAC CGA3'; P1:5'ATT ATC CGC CCC TCC GCC T3'; P2:5'AAG GGT GGT CCC CGA CCA T3'; P4:5'ATG GTC GGG GAC CAC CCT T3'; P5:5'ACC GCG CGG TTG GCC CAA AT3'; 5.8c: 5'TTG CGT TCA AAG ACT CGA TG3'; 5.8d:5'AAC CAT CGA GTC TTT GAA CGC A3'; 28cc:5'ACT CGC CGT TAC TAG GGG AA3'.

7.2.4 Generation of Restriction Fragment Length Polymorphism

7.2.4.1 Restriction Digestion of rDNA fragment

The purified rDNA was digested with the specific enzymes. 1.5 µg rDNA was digested in a 50 µl volume with *Taq* I in 100 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol (pH 8.4) supplemented with 100 µg/ml bovine serum albumin. It was incubated at 65^0C for 4 hours. The fragment was also digested with *Sau3A*I in 100 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$ (pH 7.3) supplemented with 100 µg/ml bovine serum albumin. Digestion with *Hin*fI was in 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.9). The reactions were incubated at 37^0C for 4

hours. The products were then purified by phenol extraction (Section 2.3) and DNA was precipitated by ethanol (Section 2.4). It was dissolved in 20 µl distilled water. They were resolved in a 5% PAGE and detected with silver stain.

7.2.4.2 Polyacrylamide Gel Electrophoresis (PAGE)

Mini-Protein II electrophoresis cell (BioRad) was assembled according to the manufacturer's instruction. 5% polyacrylamide gel solution was prepared by mixing 0.95 ml 40% (w/v) acrylamide solution, 0.75 ml 10XTBE, 5.8 ml water. Polymerization was initiated by adding 53 µl 10% ammonium persulfate and 3 µl TEMED. The gel solution was poured into the gap between the glass plates until it reached the top of the plates. A comb was inserted and the gel was allowed to set for 30 minutes. 20 µl of the restriction digested products were added with 4 µl 6X loading buffer and they were loaded into the wells. A constant voltage of 50V was applied. Electrophoresis was stopped when the bromophenol blue dye reached the bottom of the running gel.

7.2.4.3 Silver Staining for Nucleic Acids

BioRad Silver Staining Kit was used. The mini-vertical (8X7 cm) gel was fixed in 40% (v/v) methanol for 30 minutes and then twice in 10% (v/v) ethanol for 15 minutes.It was immersed in the silver oxidizer for 5 minutes. The gel was then rinsed in deionized water for 5 minutes twice. It was then stained in silver reagent for 20 minutes. After that, the pattern was developed in developer for about 30 seconds until the solution turned yellow or brown "smoky" precipitate appeared. Then the developer was poured off and fresh one was added. It was allowed to develop for about 5 minutes twice. The reaction was stopped in 5% (v/v) acetic acid.

7.2.5 Data Analysis

The sequences were aligned manually and the percentage identity was calculated by DNASIS. The phylogenetic trees were constructed by distance method (Section 3.2.3). The phylogenetic analysis was performed by the computer program PHYLIP.

7.3. Results and Discussion

7.3.1. rDNA Amplification and Plasmid Isolation

The fragments of 18S-ITS1-5.8S and 5.8S-ITS2-26S from six *Panax* species were cloned separately into TA vectors and transformed into *E.coli* strain INV5α. The sizes of the two fragments were about 500 and 400 bp respectively (Figs.7.2a & 7.2b). The sequences were also confirmed by direct sequencing the PCR products. The rDNA including ITS1-5.8S-ITS2, partial 18S and 26S was amplified. It was about 900 bp (Fig.7.2c). The rDNA from the adulterants *M. jalapa* L. and *P. acinosa* Roxb were subcloned and transformed into *E.coli* strain DH5α'.

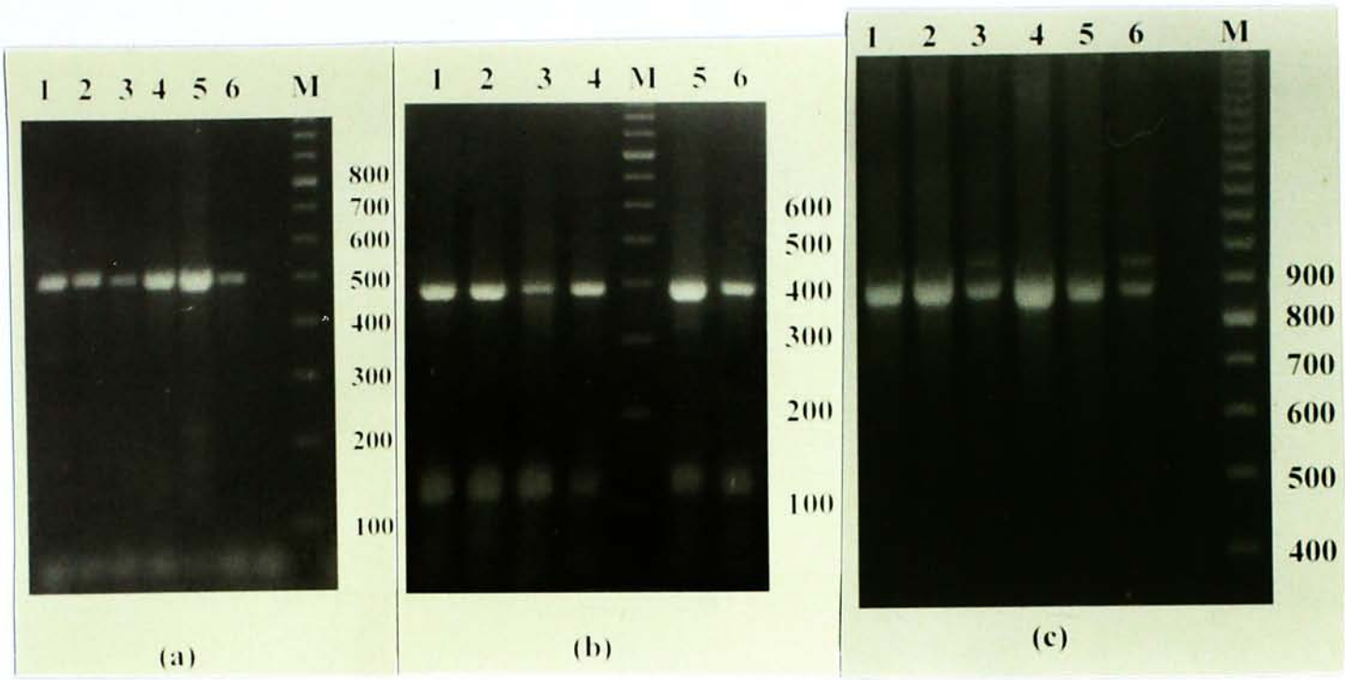

Fig.7.2: rDNA fragments amplified in *Panax* species from PCR. (a). A band of about 500 bp was observed using primers 5.8c & 18d which amplify the ITS1 region. (b). A band of about 400 bp was observed using primers 28cc & 5.8d which amplify the ITS2 region. (c). A band of about 900 bp was observed using primers 28cc & 18d which amplify the ITS1-5.8S-ITS2 region. Lane 1: *P.quinquefolius*, Lane 2: *P.ginseng*, Lane 3: *P.notoginseng*, Lane 4: *P. japonicus*, Lane 5: *P. japonicus* major, Lane 6: *P.trifolius*. M: 100 bp ladder marker.

The PCR fragments were cloned into a vector and transformed to *E.coli* (Section 2.11). White colonies in the presence of X-gal and IPTG were screened by cutting with *Eco*RI (Section 2.13).The size of the inserts were about 500 bp in ITS1, 400 bp in ITS2 and 900 bp of the rDNA bearing the two regions. They were consistent with the PCR products. Plasmid was isolated using MiniPrep (Section 2.14.1). To illustrate, results of plasmid isolation and screening by restriction digestion for the adulterants are shown in Figs 7.3 & 7.4.

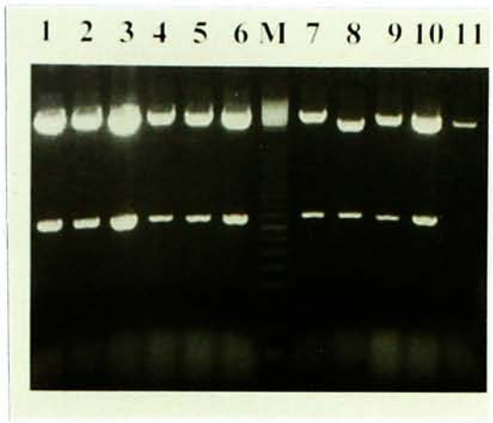

Fig.7.3: The screening of *M. jalapa* & *P. acinosa* plasmids by restriction digestion. Six colonies of each samples were screened by cutting the plasmids with *Eco*RI. A fragment of about 900 bp was released. M: 100 bp ladder marker.

Fig.7.4: The isolation of plasmid containing the insert: Colony #1 of *M. jalapa* L and colony #3 of *P. acinosa* Roxb were isolated and purified. They were then subject to sequencing. M: λ*Hin*dIII digested marker.

7.3.2 rDNA Sequencing

7.3.2.1 Sequence Comparison between the Six Panax Species and Two Adulterants

The length and GC content of rDNA from the six *Panax* species and the two adulterants are listed in Table 7.2.

	ITS1	5.8S	ITS2
1. Length (bp)			
6 *Panax* species			
i. *P. quinquefolius*	242	164	224
ii. *P. ginseng*	242	164	224
iii. *P. notoginseng*	242	164	224
iv. *P. japonicus*	240	163	224
v. *P. japonius major*	242	164	224
vi. *P. trifolius*	242	164	224
M. jalapa	196	166	209
P. acinosa	176	176	206
2. GC Content (%)			
6 *Panax* species			
i. *P. quinquefolius*	59.9	55.5	62.5
ii. *P. ginseng*	59.5	54.9	62.5
iii. *P. notoginseng*	59.9	55.5	62.5
iv. *P. japonicus*	57.9	54.3	61.6
v. *P. japonicus* major	59.9	55.2	63.4
vi. *P. trifolius*	63.2	54.3	65.2
M. jalapa	52.3	53.0	52.2
P. acinosa	55.4	55.7	59.7

Table 7.2: The characteristics of ITS1, ITS2 and 5.8S in the six *Panax* species and the two adulterants.

The sequence identity in 5.8S, ITS1 and ITS2 among the *Panax* species and the two adulterants are listed in Tables 7.3 to 7.5.

	P.Q	P.G	P.N	P.J	P.J.M	P.T
P.G	99.4					
P.N	99.4	98.8				
P.J	99.4	98.8	98.8			
P.J.M	99.4	98.8	98.8	98.8		
P.T	99.4	98.8	98.8	100	98.8	
M.J	97.0	96.4	96.4	96.4	96.4	96.4
P.A	96.0	95.5	95.5	95.5	95.5	95.5

Table 7.3: 5.8S sequence identity between six *Panax* species and two adulterants. P.Q: *P. quinquefolius*, P.G: *P. ginseng*, P.N: P. notoginseng, P.J: *P. japonicus*, P.J.M: *P. japonicus* major, P.T: *P. trifolius*.

In the 5.8S region, the percentage identity between the six *Panax* species was above 98%. The sequences were almost identical. Only one base different from *P. quinquefolius* was found in *P.ginseng, P. japonicus, P. japonicus* major and *P. trifolius*. There was no difference between *P. japonicus* and *P. trifolius*. The two adulterants had a percentage identity of 95-97% with the *Panax* species. Such a high identity demonstrated the highly conserved property of the coding region 5.8S in these different genera.

	P.Q	P.G	P.N	P.J	P.J.M	P.T
P.G	99.6					
P.N	100.0	99.6				
P.J	97.1	97.5	97.1			
P.J.M	98.8	98.3	98.8	95.9		
P.T	93.8	93.8	93.8	91.3	93.0	
M.J	55.4	56.0	55.4	55.0	51.3	55.8
P.A	59.6	58.9	59.6	54.4	58.9	61.0

Table 7.4: ITS1 region sequence identity between six *Panax* species and two adulterants. P.Q: *P. quinquefolius*, P.G: *P. ginseng*, P.N: P. notoginseng, P.J: *P. japonicus*, P.J.M: *P. japonicus* major, P.T: *P. trifolius*.

	P.Q	P.G	P.N	P.J	P.J.M	P.T
P.G	99.1					
P.N	100.0	99.1				
P.J	97.8	97.8	97.8			
P.J.M	99.1	99.1	99.1	97.8		
P.T	94.6	94.6	94.6	94.2	95.5	
M.J	56.0	56.0	56.0	52.9	55.5	56.5
P.A	63.2	63.2	63.2	62.6	63.2	50.9

Table 7.5: ITS2 region sequence identity between six *Panax* species and two adulterants.

Variations appeared in the internal transcribed spacers. Fig.7.5 shows the aligned sequences of the species. In the ITS1 region, one to fifteen bases difference between *P. quinquefolius* and other *Panax* species was found. On the other hand, one to twelve bases difference was present in the ITS2 region. It was noticed that the base differences in the ITS regions were mainly due to transition which occupied about 70% of the base changes. The rest of them was resulted from transversion. Fig.7.6 shows the base difference between *P. quinquefolius* and *P.ginseng* in the ITS1 region.

The sequence identity of the internal transcribed regions among the *Panax* species were very high. Most of them were above 97% except *P. trifolius* which was less related to other species. *P. quinquefolius* was identical to *P. notoginseng* and was almost equidistant from *P. ginseng*. *P. japonicus* was also less related to other *Panax* as it had about 97% identity in ITS1 and ITS2 regions; while other has an average of above 98% identity.

```
P.Q        1 TCGGAGCGCA CGTCGAGGAC GGCGCAACAG GGTCATGAGA GCTTTTGCTG  50
P.G        1 ********** ********** ********** ********** **********  50
P.N        1 ********** ********** ********** ********** **********  50
P.J        1 ********** ********** ********** ********** *******T**  50
P.J.M      1 *********G ********** ********** ********** **********  50
P.T        1 *********G *****G**** ********** ****G***** **C*****C*  50

P.Q       51 GCGACGGGTC ACCGCACGAC ATGAGAAGAG GGCTTTTTAC AACCACCACT 100
P.G       51 ********** ********** ********** ********** ********** 100
P.N       51 ********** ********** ********** ********** ********** 100
P.J       51 ****A***** ********** ********** ********** ********** 100
P.J.M     51 ********** ********** ********** ********** ********** 100
P.T       51 ********** ********** T********* ********** ********** 100

P.Q      101 TGTCGTGACG TCCATCGCCA AGGACTCGCA TTTGGGCCAA CCGCGCGGTG 150
P.G      101 ********** ********** ********** ********** ********** 150
P.N      101 ********** ********** ********** ********** ********** 150
P.J      101 ********** ********** ********** ********** ****A***** 150
P.J.M    101 ********** ********** ********** ********** ********** 150
P.T      101 ********** ***G*****G ********** ********** *********T 150

P.Q      151 AGACACGGGA GGCCATTATC CGCCCCTCCG CCTCGACTCC CGCAAAGGAG 200
P.G      151 ********** ********** ********** ****A***** *****G**** 200
P.N      151 ********** ********** ********** ********** ********** 200
P.J      151 ********** *****A**** ********** ********** *****G**** 200
P.J.M    151 ********** ********** ********** ********** *****G**** 200
P.T      151 ********** *****A**** ********** ********** **T**G**** 200

P.Q      201 TGATGGGTTG GGGGGCGACG CGATGCGTGA CGCCCAGGCA GACGTGCCCT  250
P.G      201 ********** ********** ********** ********** **********  250
P.N      201 ********** ********** ********** ********** **********  250
P.J      201 ********** ********** ********** ********** **********  250
P.J.M    201 ********** ********** ********** ****-***** **********  250
P.T      201 ********** ********** ********** ********** **********  250

P.Q      251 CGGCCTAATG GCTTCGGGCG CAACTTGCGT TCAAAGACTC GATGGTTCAC  300
P.G      251 ********** ********** ********** ********** **********  300
P.N      251 ********** ********** ********** ********** ****A*****  300
P.J      251 ********** ****A***** ********** ********** **********  300
P.J.M    251 ********** ********** ********** ********** **********  300
P.T      251 ********** ****A***** ********** ********** **********  300

P.Q      301 GGGATTCTGC AATTCACACC AAGTATCGCA TTTCGCTACG TTCTTCATCG  350
P.G      301 ********** ********** ********** ********** **********  350
P.N      301 ********** ********** ********** ********** **********  350
P.J      301 ********** ********** ********** ********** **********  350
P.J.M    301 ********** ********** ********** ********** **********  350
P.T      301 ********** ********** ********** ********** **********  350
```

```
P.Q      351 ATGCGAGAGC CGAGATATCC GTTGCCGAGA GTCGTTTGTG TTTTAGAAAG   400
P.G      351 ********** ********** ****T***** ********** **********   400
P.N      351 ********** ********** ********** ********** **********   400
P.J      351 ********** ********** ********** ********** **********   400
P.J.M    351 ********** ********** ********** ********** **********   400
P.T      351 ********** ********** ********** ********** **********   400

P.Q      401 ACGCTTCCGC CGCCCGCAAA CGGGGGGGAC GCGTGCAGTT CAGTTTGATT   450
P.G      401 ********** ********** ********** ********** **********   450
P.N      401 ********** ********** ********** ********** **********   450
P.J      401 ********** ********** T********* ********** **********   450
P.J.M    401 ********** ********** ********** ********** **********   450
P.T      401 ********** ********** ********** ********** **********   450

P.Q      451 TCCTTGGCGC ATTCCGCGCC GGGGGGTCGT TGTTCGGACG AGAGCCACCC   500
P.G      451 ********** ********** ********** ********** ***T******   500
P.N      451 ********** ********** ********** ********** **********   500
P.J      451 ********A* ********** ********** ********** ***T*****-   500
P.J.M    451 ********** ********** ********** ********** **********   500
P.T      451 ********** ********** ********** ********** G*GAG*****   500

P.Q      501 AAGGGTGGTC CCCGACCATG GGTTTGCAAC TTGGGGAGCT TGCGCACCCC   550
P.G      501 ********** ********** ********** ********** **********   550
P.N      501 ********** ********** ********** ********** **********   550
P.J      501 *******-** ********** ********** ********** *******G**   550
P.J.M    501 **T*****A* ********** ********** ********** **********   550
P.T      501 GG***C**C* ********** ****C*G*** ******G*** *********T   550

P.Q      551 TCGTCCCTCA CCCGGTATTG TAACGTGTTC GCGGGTCGTT CTGCTATGCA   600
P.G      551 ********** ********** ********** ********** **********   600
P.N      551 ********** ********** ********** ********** **********   600
P.J      551 ********** ********** ********** A********* **********   600
P.J.M    551 ********** *******G** ********** ********** **********   600
P.T      551 ********** ******G*** A********* ********** *****G****   600

P.Q      601 GGTTTCGACA ATGATCCTTC CGCAGGTTCA 630
P.G      601 ********** ********** ********** 630
P.N      601 ********** ********** ********** 630
P.J      601 ********** ********** ********** 630
P.J.M    601 ********** ********** ********** 630
P.T      601 ********** ********** ********** 630
```

□

Fig.7.5: The aligned ITS2-5.8S-ITS1 sequences of six *Panax* species. The sequences were aligned manually in the DNASIS program. P.Q: *P. quinquefolius*; P.G: *P. ginseng*; P.N: *P. notoginseng*; P.J: *P. japonicus*; P. J.M: *P. japonicus* major; P. T: *P. trifolius.* "*" denotes nucleotide that matches with *P. quinquefolius.*"-" denotes gap that is inserted. The coding region 5.8S is highlighted. The ITS2 region starts at nucleotide 1 to 224 and the ITS1 region starts at nucleotide 388 to 630.

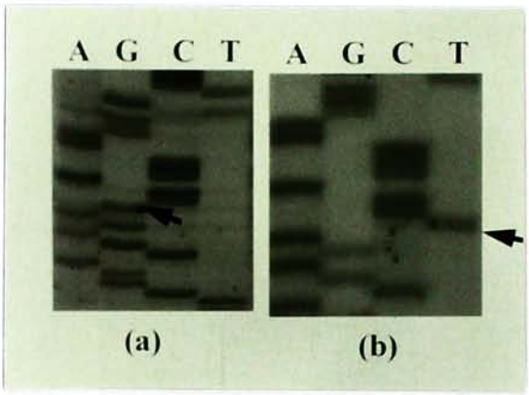

Fig.7.6: The sequence differences between (a) *P.quinquefolius* and (b) *P. ginseng*. Arrow indicates the base difference that enables the differentiation of them.

When comparing the two adulterants, both ITS1 and ITS2 had great differences. The length of the ITS1 and ITS2 in *M. jalapa* and *P. acinosa* are shown in Table 7.2. When compared with the six *Panax* species, the ITS regions were about 55 to 61 % identity to them (Tables 7.4 & 7.5).

7.3.3 Restriction Fragment Length Polymorphism

7.3.3.1 Restriction Profiles between Ginsengs and their Adulterants

Three enzymes were used to generate unique fingerprints for *P. quinquefolius and P. ginseng* as well as their adulterants. Moreover, different proportion of both ginseng DNA were also included to reveal patterns of the mixture. The patterns are shown in Figs.7.7 to 7.9. The restriction sites of the enzymes and the expected fragments produced are summarized in Table 7.3.

	P. quinquefolius	***P. ginseng***
***Hinf*I (GCG!C)**	18 220 282 383 400 476	18 220 383 400 476
Fragments produced (in bp)	**17 18 62 76 101 202 404**	**17 18 76 163 202 404**
***Sau*3AI (!GATC)**	710	589 710
Fragments produced (in bp)	**171 710**	**121 170 589**
***Taq*I (T!CGA)**	110 280 386 445 702 789	110 386 445 472 702 789
Fragments produced (in bp)	**59 87 106 110 170 257**	**27 59 87 110 230 276**

Table 7.3: A table showing the coordinates for cleavage to occurr and the fragments produced in both ginsengs.

Three polymorphic bands (Fig.7.7), #1 and #2 of 62 and 101 bp in *P. quinquefolius* and #3 of 163 bp in *P. ginseng* generated by *Hin*fI could act as the marker to distinguish between both ginseng. Moreover, two obvious bands (Fig.7.8) #1 of 121 bp and #2 of 589 bp generated by *Sau*3AI in *P. ginseng* also enabled the differentiation of these two species. In the *Taq*I restriction profile (Fig.7.9), although two fragments #1 and #2 of 106 and 110 bp in *P. quinquefolius* were of little difference in molecular weight, they could still be resolved in polyacrylamide gel. In addition, the fragments #3 and #4 of 170 and 257 bp in *P. quinquefolius* and fragments #5 and #6 of 230 and 276 bp in *P. ginseng* were easily identified in the polyacrylamide gel. These highly polymorphic patterns could be used to distinguish both ginsengs.

When comparing the profile of the adulterants generated by *Hin*fI (Fig.7.7), the fragments of about 250 and 350 bp were unique to *M. jalapa*. The adulterant *P. acinosa* could not be distinguished from *P. ginseng* as they had the same restriction pattern. In the *Sau*3AI restriction pattern (Fig.7.8), two bands below 100 bp were detected in *M. jalapa* and one fragment in *P. acinosa*. By comparing the marker bands in both ginseng and the polymorphic bands in the adulterants, it was able to distinguish them. Furthermore, profile of the ginseng mixtures showed patterns characteristics of both ginsengs, even *P. quinquefolius* was present in one-tenth of *P. ginseng* or vice versa.

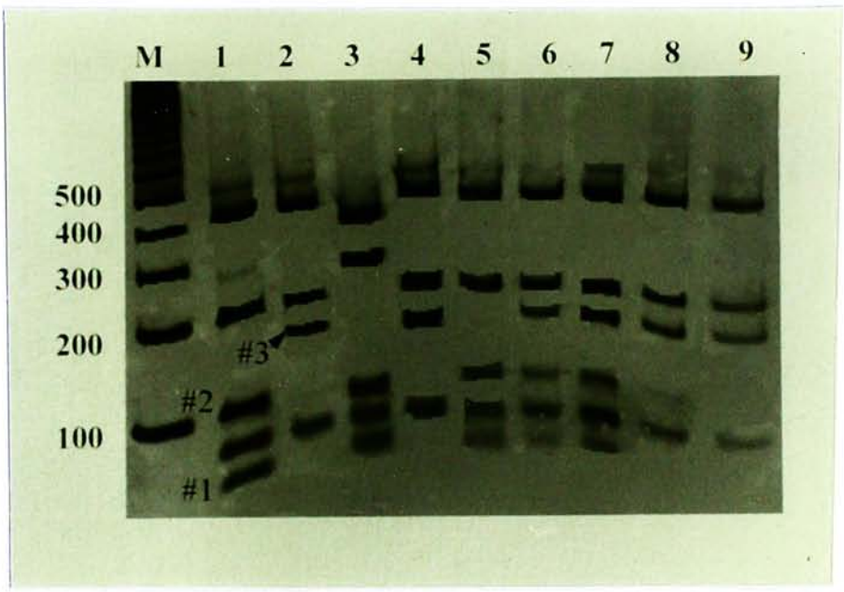

Fig.7.7: The RFLP pattern of both ginsengs and their adulterants generated by *Hin*fI. The restriction fragments were resolved on a 5% polyacrylamide gel and stained with silver reagent. Lane 1: *P. quinquefolius*, Lane 2: *P. ginseng*, Lane 3: *M. jalapa* L, Lane 4: *P. acinosa* Roxb, Lane 5: Mixtures of both *P. quinquefolius* and *P. ginseng* in proportion of 9:1, Lane 6: Mixtures of both *P. quinquefolius* and *P. ginseng* in proportion of 7:3,Lane 7: Mixtures of both *P. quinquefolius* and *P. ginseng* in proportion of 1:1, Lane 8: Mixtures of both *P. quinquefolius* and *P. ginseng* in proportion of 3:7, Lane 9: Mixtures of both *P. quinquefolius* and *P. ginseng* in proportion of 1:9. M: 100 bp ladder marker

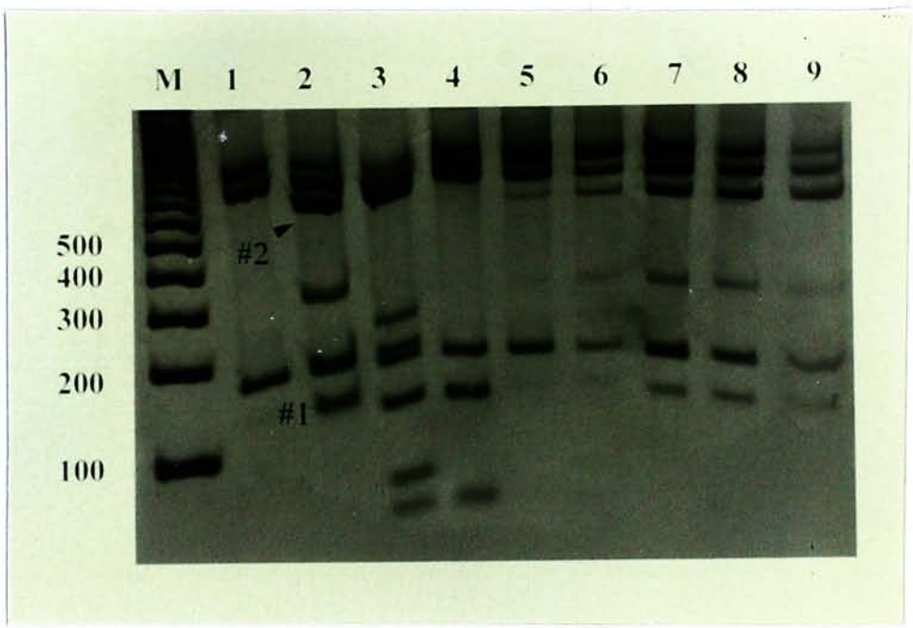

Fig.7.8: The RFLP pattern of both ginsengs and their adulterants generated by *Sau*3AI. The restriction fragments were resolved on a 5% polyacrylamide gel and stained with silver reagent. Lane 1: *P. quinquefolius*, Lane 2: *P. ginseng*, Lane 3: *M. jalapa* L, Lane 4: *P. acinosa* Roxb, Lane 5: Mixtures of both *P. quinquefolius* and *P. ginseng* in proportion of 9:1, Lane 6: Mixtures of both *P. quinquefolius* and *P. ginseng* in proportion of 7:3,Lane 7: Mixtures of both *P. quinquefolius* and *P. ginseng* in proportion of 1:1, Lane 8: Mixtures of both *P. quinquefolius* and *P. ginseng* in proportion of 3:7, Lane 9: Mixtures of both *P. quinquefolius* and *P. ginseng* in proportion of 1:9. M: 100 bp ladder marker.

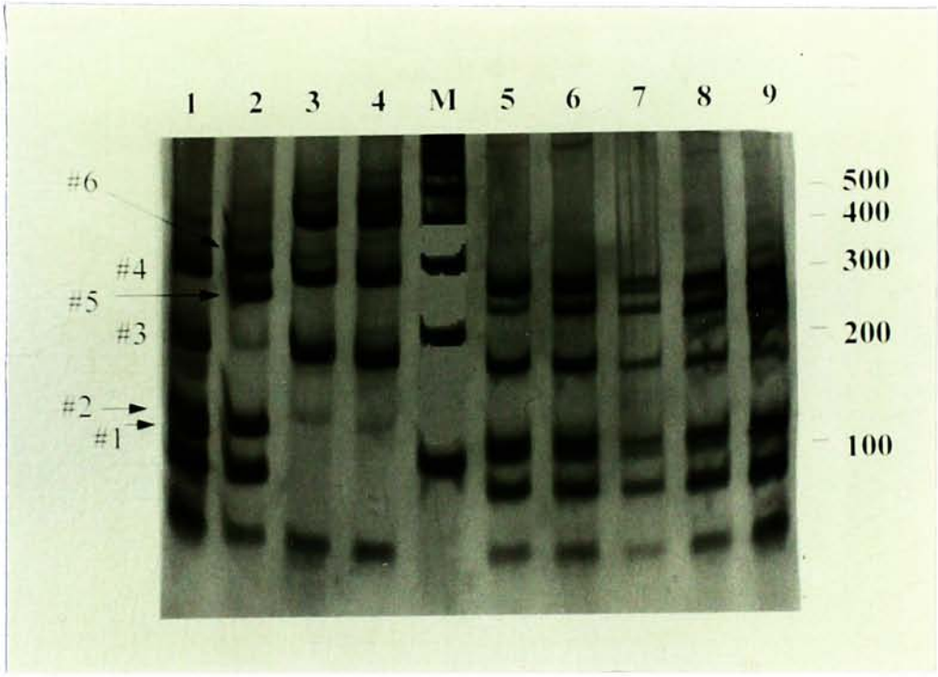

Fig.7.9: The RFLP pattern of both ginsengs and their adulterants generated by *Taq*I. The restriction fragments were resolved on a 5% polyacrylamide gel and stained with silver reagent. Lane 1: *P. quinquefolius*, Lane 2: *P. ginseng*, Lane 3: *M. jalapa* L, Lane 4: *P. acinosa* Roxb, Lane 5: Mixtures of both *P. quinquefolius* and *P. ginseng* in proportion of 9:1, Lane 6: Mixtures of both *P. quinquefolius* and *P. ginseng* in proportion of 7:3,Lane 7: Mixtures of both *P. quinquefolius* and *P. ginseng* in proportion of 1:1, Lane 8: Mixtures of both *P. quinquefolius* and *P. ginseng* in proportion of 3:7, Lane 9: Mixtures of both *P. quinquefolius* and *P. ginseng* in proportion of 1:9. M: 100 bp ladder marker.

7.3.3.2 Restriction Profiles of Ginseng from Different Sources

In order to investigate polymorphism exists in *Panax* species from various sources, two *P. quinquefolius* from USA and Canada respectively and one *P. ginseng* from Russia were compared with the *P. quinquefolius* from Canada and *P. ginseng* from China by RFLP analysis. Figs.7.10 to 7.12 show the restriction profiles of the ginsengs from different locations. No polymorphism was detected by *Hin*fI and *Sau*3A. However, one fragment of about 250 bp was generated in *P.ginseng* from Russia using enzyme *Taq* I (Fig.7.12). It was not surprising because no *Taq* I cutting site (T!CGA) was found at nucleotide 472 in the sequence of *P. ginseng* from Russia, where *P. ginseng* from China did have (Fig.7.13). Therefore, for precise authentication more than one enzyme should be used for RFLP study.

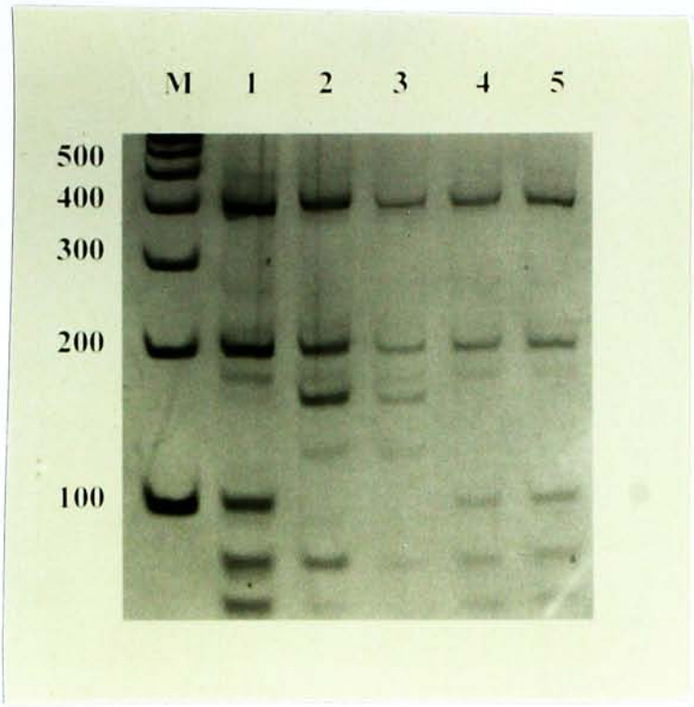

Fig.7.10: The RFLP pattern of ginseng from different locations using *Hin*fI. The restriction fragments were resolved on a 5% polyacrylamide and stained with silver reagent. Lane 1: *P. quinquefolius* from Canada, Lane 2: *P. ginseng* from China, Lane 3: *P.ginseng* from Russia, Lane 4: *P. quinquefolius* from USA, Lane 5: *P. quinquefolius* from Canada. M: 100 bp ladder marker.

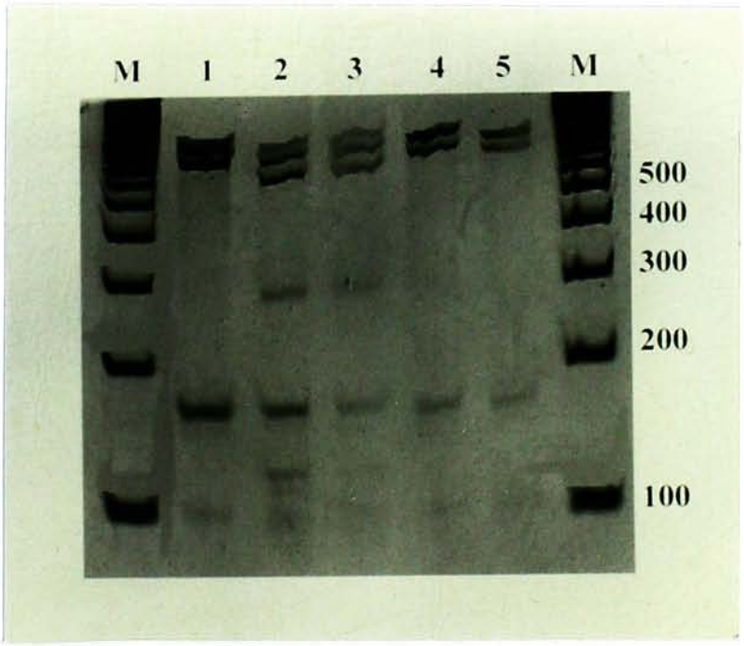

Fig.7.11: The RFLP pattern of ginsengs from different locations using *Sau*3A. The restriction fragments were resolved on a 5% polyacrylamide and stained with silver reagent. Lane 1: *P. quinquefolius* from Canada, Lane 2: *P. ginseng* from China, Lane 3: *P.ginseng* from Russia, Lane 4: *P. quinquefolius* from USA, Lane 5: *P. quinquefolius* from Canada. M: 100 bp ladder marker.

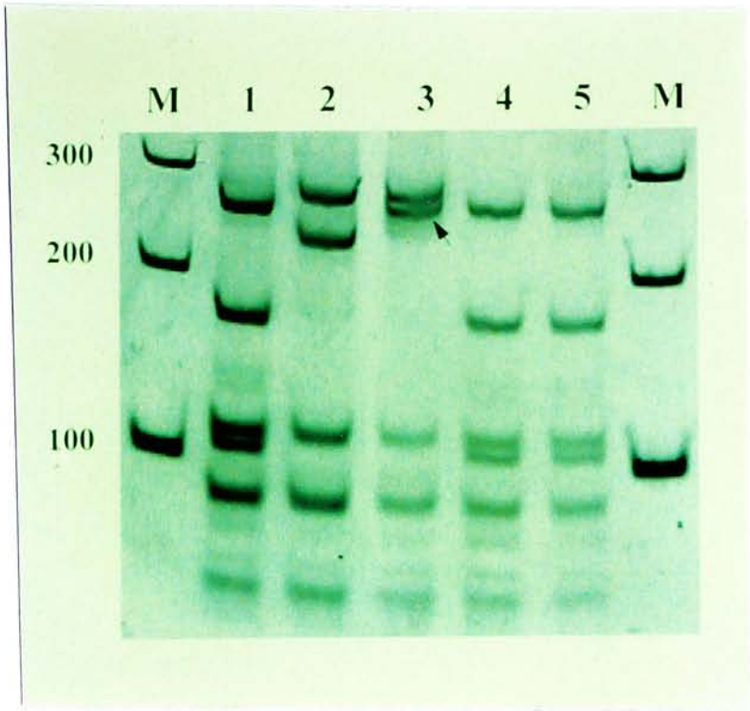

Fig.7.12: The RFLP pattern of ginseng from different locations using *Taq*I. The restriction fragments were resolved on a 5% polyacrylamide and stained with silver reagent. Lane 1: *P. quinquefolius* from Canada, Lane 2: *P. ginseng* from China, Lane 3: *P.ginseng* from Russia, Lane 4: *P. quinquefolius* from USA, Lane 5: *P. quinquefolius* from Canada. M: 100 bp ladder marker. Arrow indicates the band that is different from *P. ginseng* from China

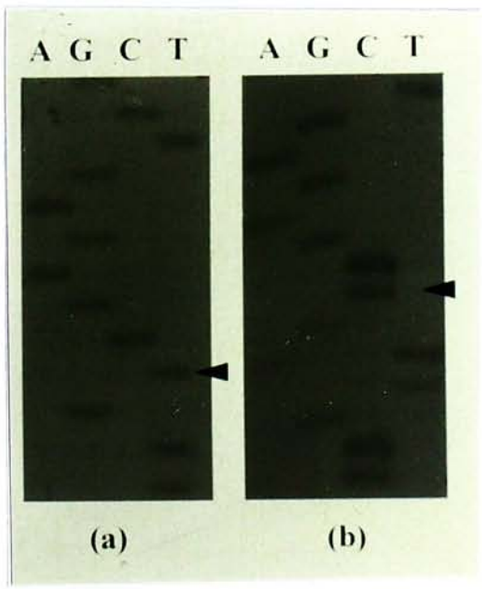

Fig.7.13: The sequence differences between *P. ginseng* from (a) Russia and (b) China. Arrow indicates the base change that results in different RFLP pattern.

7.3.4. Panax Phylogeny

The relatedness of the six *Panax* species was also estimated. As indicated in Table 7.3, the percentage identity in the 5.8S region of the six *Panax* species was almost the same. Thus it was not included in the phylogenetic studies. Figs.7.14 & 7.15 illustrate the relationship among the *Panax* species in ITS1 and ITS2, respectively. The results showed that P. *trifolius* was an outgroup since it had the lowest identity with the other *Panax* species. On the other hand, *P. quinquefolius* and *P. ginseng* were not the most closely related species. Instead, *P. notoginseng* was closely related to *P. quinquefolius*. This was in discrepancy to the morphological or chemical as well as random-primed PCR analysis. Such discrepancy might be attributed to the limited variation in the rDNA. In the two ITS regions, there were altogether three bases changed in the nucleotides between *P. quinquefolius* and *P. ginseng*. It represented only a 0.6% difference. Moreover, the sequences of ITS regions in *P. quinquefolius* and *P. notoginseng* were almost identical, with only one base difference. Since these three species were highly homologous to each other, it made the analysis inaccurate.

It was also surprising to find that *P. japonicus* major was more related to *P. quinquefolius* and *P.notoginseng*. It was supposed to be more related to *P. japonicus* as both of them have horizontal rhizomes. The highly conserved rDNA could not produce significant difference to reveal a true phylogeny in the various *Panax* species. Other regions such as intergenic spacers (IGS) which separates the repeated unit may be worth trying to study the relationship between the different *Panax* species. It is because this region evolves so rapidly (Porter and Collins, 1991) and the difference between closely related species may be more significant.

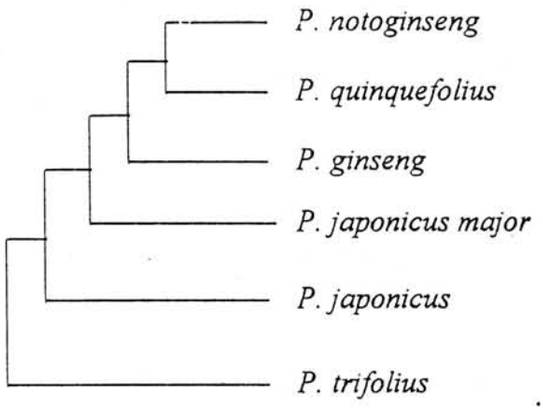

Fig.7.14: The phylogenetic tree generated using sequence from ITS1 region of six *Panax* species.

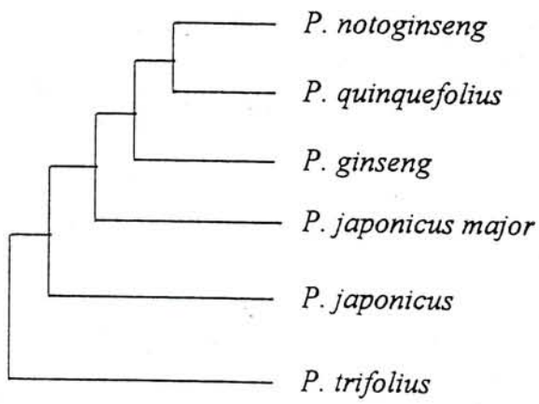

Fig.7.15: The phylogenetic tree generated using sequence from ITS2 region of six *Panax* species.

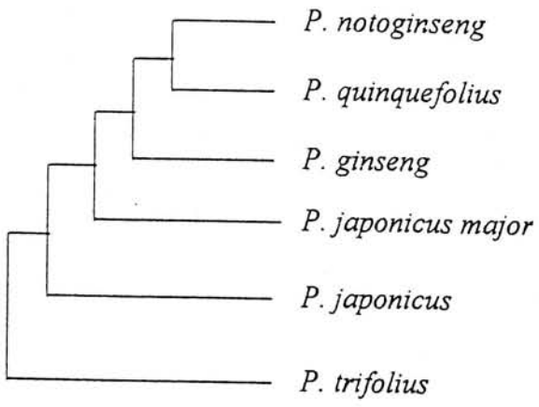

Fig.7.16: A consensus tree showing the relationship among the six *Panax* species.

Chapter 8 General Discussion

Chapter 8 General Discussion

8.1 Advantages of Random-Primed PCRs

RAPD or AP-PCR profilings are being increasingly used in population surveys because of the ease of methodology and the numerous distinguishable polymorphisms. Those bands have been shown to be inherited in Mendelian fashion, and therefore useful as molecular markers for qualitative and quantitative traits (Williams *et al*, 1990; Hadry *et al*, 1992). There are a number of advantages of using such profiling in authentication.

Firstly, small amount of sample DNA, as low as 10 ng, is sufficient for generating a DNA fingerprint. It is particular useful in Chinese medicinal plants because some of them are expensive and DNA may be degraded during processing.

Secondly, the procedure of setting up reaction is just simply by mixing all the reagents and DNA template. The products are resolved by electrophoresis and detected by fluorescence. No radioistope is used. The procedure is simple and data can be obtained within one day. It speeds up the analysis process. Thus it poses the possibility in providing services in the herbal industry.

Thirdly, the patterns are not affected by the age, sources and physiological conditions of the plants as well as the environmental factors. The results could be more accurate.

Fourthly, the method is not restricted to the physical forms of the herbs unless DNA can be extracted. Some of the products like ginseng powder are difficult to identify by direct examining the morphology.

Fifthly, the primers used in the random-primed PCRs are commercially available or any primers in the laboratory can also be used for generating fingerprints. It is relatively

cheaper and saves times as compared to RFLP or isozymes analysis, in which various enzymes might have to be tested to reveal polymorphisms.

8.2 Weakness of the Random-Primed PCRs

One of the errors in the random-primed PCRs is the assumption that co-migrating RAPDs are amplified from the same genomic locus and that two genotypes sharing the absence of a RAPD do so because of similarity at that RAPD locus. In addition, it is assumed that the level of sequence similarity between genomes indicated by monomorphic comparisons and the amount of sequence difference indicated by presence versus absence of RAPD amplified are the same for all comparisons. Violation of these assumption would lead to an increase in the variance of genetic distance estimates. Thus such profiling is more appropiate in studying relationship between closely related species.

Random-primed PCRs are very sensitive techniques, reproducibility might sometimes be a problem. Changes in template DNA concentration by half could result in different number of bands. Different thermocycler may also have influence in producing the banding patterns. There is a major effect of the ramp time from the annealing step to the extension step (Klein-Lankhorst *et al*, 1991; Bruno *et al*, 1993). The longer it takes for the temperature to change from 35^0C to 72^0C, the more primers give reproducible, robust fingerprints.

Apart from that, scoring error might also has an impact on estimating genetic distance. Scoring are mainly dependent on the intensity of the bands stained with ethidium bromide. In order to minimize such error, only distinct and reproducible bands were scored in the herbs studied. Other weakly amplified or 'sporadic' bands were eliminated in this study. The weak amplification of some RAPD bands compared to others may reflect differences in the specificity of amplification and underlying differences in the level of

sequence polymorphism that each type of RAPD represents. However, they might also be the result of primer mismatch.

It is known that different loci may have different genetic information. Thus primer binds on various loci may show different extent of homology. It was seen in the genetic distances calculated in the *Acorus* and *Epimedium* species. The accurracy of the genetic distance could be increased by using more primers, for example, 20 to 50 primers or until a phylogenetic tree that does not have any changes in the cluster formation is found.

8.3 Molecular Markers for Phylogenetic Studies

RAPD/AP-PCR profiling are more relevant to identify different herbs rather than for phylogenetic studies on the ground that information on the bands is still an unknown. A clear picture of phylogeny can be achieved by sequencing regions like rDNA or the large subunit of ribulose-1,5-bisphosphate carboxylase (rbcL) which have been well documented in phylogenetic studies in seed plants (Chase *et al*, 1993).

8.4 Specific PCR-RFLP Patterns in Authentication

PCR-RFLP is a precise authentication tool. It eliminates the problem of reproducibility in generating DNA fingerprints in random-primed PCRs. The number of bands and patterns are definite and unique to each species. It can also detect polymorphisms between different individuals of the same species. It provides a remarkable technique in authentication of Chinese medicinal herbs.

Future work can be done using microsatellites or simple repetitive sequences as an alternative source of genetic markers, which have been reported of highly polymorphic in

wheat (Röder *et al*, 1995). Moreover, the distinct bands in each species could be isolated and sequenced to make probes for future identification.

8.5 Conclusions

Molecular techniques of random-primed PCRs and PCR-RFLP were successfully used to distinguish different *Panax* species. Furthermore, ten commercial ginseng products were assessed to determine the composition. Seven of them matched the conclusion from HPLC, but three samples could not be identified. This may be due to the sensitivity of the techniques that detected the presence of low level of other materials, or the processed samples which had the property of the DNA changed. Unique fingerprints were also generated for differentiating various *Acorus* and *Epimedium* species.

The phylogeny of *Panax* species from the random-primed PCRs concluded that *P. quinquefolius* was more closely related to *P. ginseng* than *P. notoginseng*. It was in accordance with morphological and chemical analysis. However, the sequence of ITS1 and ITS2 suggested that *P. quinquefolius* was more related to *P. notoginsseng*, but not *P. ginseng*. It might be due to a high homology in the sequenced region between *P. quinquefolius, P. ginseng* and *P. notoginseng* that cannot reveal a true phylogeny. On the other hand, sequencing of the rDNA has allowed us to explore the polymorphism present and use it as an effective tool in authentication. Moreover, the extent of variation in the *Panax* genome has not been known. Therefore, it provides a guideline to the forecoming research work.

By employing these molecular approaches, we hope that a library of various Chinese medicinal plant sequences and unique fingerprints either from RAPD/AP-PCR or PCR-RFLP can be established to provide a more accurate and specific way in authentication in complement with other methods.

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Appendix

Appendix I

A list of potent herbs controlled in China (C) and Taiwan (T).

1. rootstock of *Aconitum carmichaeli* (C,T)
2. rootstock of *Aconitum kusnezoffii* (C,T)
3. lateral root of *Aconitum carmichaeli* (C)
4. root-tuber of *Aconitum brachypodum* (C)
5. root of *Aconitum coreanum* (T)
6. root of *Euphorbia ebracteolata* and *E. fischeriana* (C,T)
7. root of *Knoxia valerianoides* (T)
8. root of *Phytolacca acinosa* and *P. americana* (T)
9. tuber of *Arisaema erubescens*, *A. heterophyllum* and *A. amurense* (C,T)
10. tuber of *Euphorbia kansui* (C,T)
11. tuber of *Pinellia ternata* (C)
12. tuber of *Typhonium giganteum* (C)
13. stem bark and root bark of *Melia azedarach* and *M. toosendan* (T)
14. resin of *Garcinia morella* (C)
15. resin of *Phus vermciflua* (T)
16. flower of *Datura metel* (T)
17. flower bud of *Rhododendron molle* (C)
18. flower bud of *Daphne genkwa* (T)
19. fruit of *Croton tiglium* (C,T)
20. seed of *Euphorbia lathyris* (C,T)
21. seed of *Hyoscyamus niger* (C,T)
22. seed of *Impatiens balsamina* (T)
23. seed of *Pharbitis nil and P. purpurea* (T)
24. seed of *Ricinus communis* (T)
25. seed of *Strychnos pierriana* and *S. nux-vomica* (C,T)
26. body of *Buthus martensii* (T)
27. body of *Hirudo nipponica, Whitmania pigra* and *W. acranulata* (T)
28. body of *Huechys sanguinea* and *H. philaemata* (C)
29. body of *Lytta caraganae* (C)
30. body of *Mylabris phalerata* and *M. cichorii* (C,T)
31. body of *Scolopendra subspinipes mutilans* (T)
32. body of *Tabanus bivittatus* (T)
33. secretion of *Bufobufo gargarizans* and *B. melanostictus* (C,T)
34. skin slough of *Elaphe carinata*, *E. taeniura* and *Zaocys dhumnades* (T)
35. arsenic (C)
36. arsenolite (C,T)
37. calomel (C,T)
38. chalcanthite (T)
39. crystal formed with mercurous chloride and mercuric chloride (C)

40. mercuric oxide and processed mixture of mercury, niter and alunite (C)
41. mercury (C)
42. realgar (C,T)
43. sal-ammoniac (T)

Appendix II

RAPD Primers

Primer Code	5' to 3'
OPC-01	TTCGAGCCAG
OPC-01	GTGAGGCGTC
OPC-03	GGGGGTCTTT
OPC-04	CCGCATCTAC
OPC-05	GATGACCGCC
OPC-06	GAACGGACTC
OPC-07	GTCCCGACGA
OPC-08	TGGACCGGTG
OPC-09	CTCACCGTCC
OPC-10	TGTCTGGGTG
OPC-11	AAAGCTGCGG
OPC-12	TGTCATCCCC
OPC-13	AAGCCTCGTC
OPC-14	TGCGTGCTTG
OPC-15	GACGGATCAG
OPC-16	CACACTCCAG
OPC-17	TTCCCCCCAG
OPC-18	TGAGTGGGTG
OPC-19	GTTGCCAGCC
OPC-20	ACTTCGCCAC
OPF-01	ACGGATCCTG
OPF-02	GAGGATCCCT
OPF-03	CCTGATCACC
OPF-04	GGTGATCAGG
OPF-05	CCGAATTCCC
OPF-06	GGGAATTCGG
OPF-07	CCGATATCCC
OPF-08	GGGATATCGG
OPF-09	CCAAGCTTCC
OPF-10	GGAAGCTTGG
OPF-11	TTGGTACCCC
OPF-12	ACGGTACCAG
OPF-13	GGCTGCAGAA
OPF-14	TGCTGCAGGT
OPF-15	CCAGTACTCC
OPF-16	GGAGTACTGG
OPF-17	AACCCGGGAA

OPF-18	TTCCCGGGTT
OPF-19	CCTCTAGACC
OPF-20	GGTCTAGAGG
OPAA-01	AGACGGCTCC
OPAA-02	GAGACCAGAC
OPAA-03	TTAGCGCCCC
OPAA-04	AGGACTGCTC
OPAA-05	GGCTTTAGCC
OPAA-06	GTGGGTGCCA
OPAA-07	CTACGCTCAC
OPAA-08	TCCGCAGTAG
OPAA-09	AGATGGGCAG
OPAA-10	TGGTCGGGTG
OPAA-11	ACCCGACCTG
OPAA-12	GGACCTCTTG
OPAA-13	GAGCGTCGCT
OPAA-14	AACGGGCCAA
OPAA-15	ACGGAAGCCC
OPAA-16	GGAACCCACA
OPAA-17	GAGCCCGACT
OPAA-18	TGGTCCAGCC
OPAA-19	TGAGGCGTGT
OPAA-20	TTGCCTTCGG
OPAM-01	TCACGTACGG
OPAM-02	ACTTGACGGG
OPAM-03	CTTCCCTGTG
OPAM-04	GAGGGACCTC
OPAM-05	GGGCTATGCC
OPAM-06	CTCGGGATGT
OPAM-07	AACCGCGGCA
OPAM-08	ACCACGAGTG
OPAM-09	TGCCGGTTCA
OPAM-10	CAGACCGACC
OPAM-11	AGATGCGCGG
OPAM-12	TCTCACCGTC
OPAM-13	CACGGCACAA
OPAM-14	TGGTTGCGGA
OPAM-15	GATGCGATGG
OPAM-16	TGGCGGTTTG
OPAM-17	CCTAACGTCC
OPAM-18	ACGGGACTCT
OPAM-19	CCAGGTCTTC
OPAM-20	ACCAACCAGG

OPAW-01	ACCTAGGGGA
OPAW-02	TCGCAGGTTC
OPAW-03	CCATGCGGAG
OPAW-04	AGGAGCGACA
OPAW-05	CTGCTTCGAG
OPAW-06	TTTGGGCCCC
OPAW-07	AGCCCCCAAG
OPAW-08	CTGTCTGTGG
OPAW-09	ACTGGGTCGG
OPAW-10	GGTGTTTGCC
OPAW-11	CTGCCACGAG
OPAW-12	GAGCAAGGCA
OPAW-13	CTACGATGCC
OPAW-14	GGTTCTGCTC
OPAW-15	CCAGTCCCAA

AP-PCR Primers

Primer Code	**5' to 3'**
GalK	TACGGTGGCGGAGCGCAGCA
M13 forward	CGCCAGGGTTTTCCCAGTCACGAC
M13 backward	AGCGGATAACAATTTCACACAGGA
Seq2	CTGGTCAAGGCACAAGAGAT
Str2 forward	TGGGGCATATGTTCATGCCGCCT
Str2 backward	TTGGATCCTAGTATGACGTCTG
TCS forward	GTTTTCCATATGAATCTGAGAAAAGCT
TCS backward	GGTGGATCCCTAAGCATCAACATTGGT
Primer#10	GCCTATCAAAAGCAGAT
Primer#13	ATGAGCCATGGCAGTGAGTATCGC
Primer#14	ATTCAGGATCTTGATAAATGAGTT
Primer#15	AAGCCCATATGAAAAAGAAACCA
Primer#16	TTTTGCGTATGTATAAAA
Primer#17	TTTTATACATACGCAAAACTGATAGAAGAA
Primer#18	CATCGGATCCACCACGTC
Primer#20	GCCCCCTTTATCAACGATTCT

Appendix III

Comments for pCR™II
3932 nucleotides

*Lac*Z gene: bases 1-571
Sp6 promoter: bases 239-255
Multiple Cloning Site: bases 269-381
T7 promoter: bases 388-407
F1 origin: bases 572-986
Kanamycin resistance: bases 987-2114
Ampicillin resistance: bases 2133-2992
ColE1 origin: bases 3182-3765

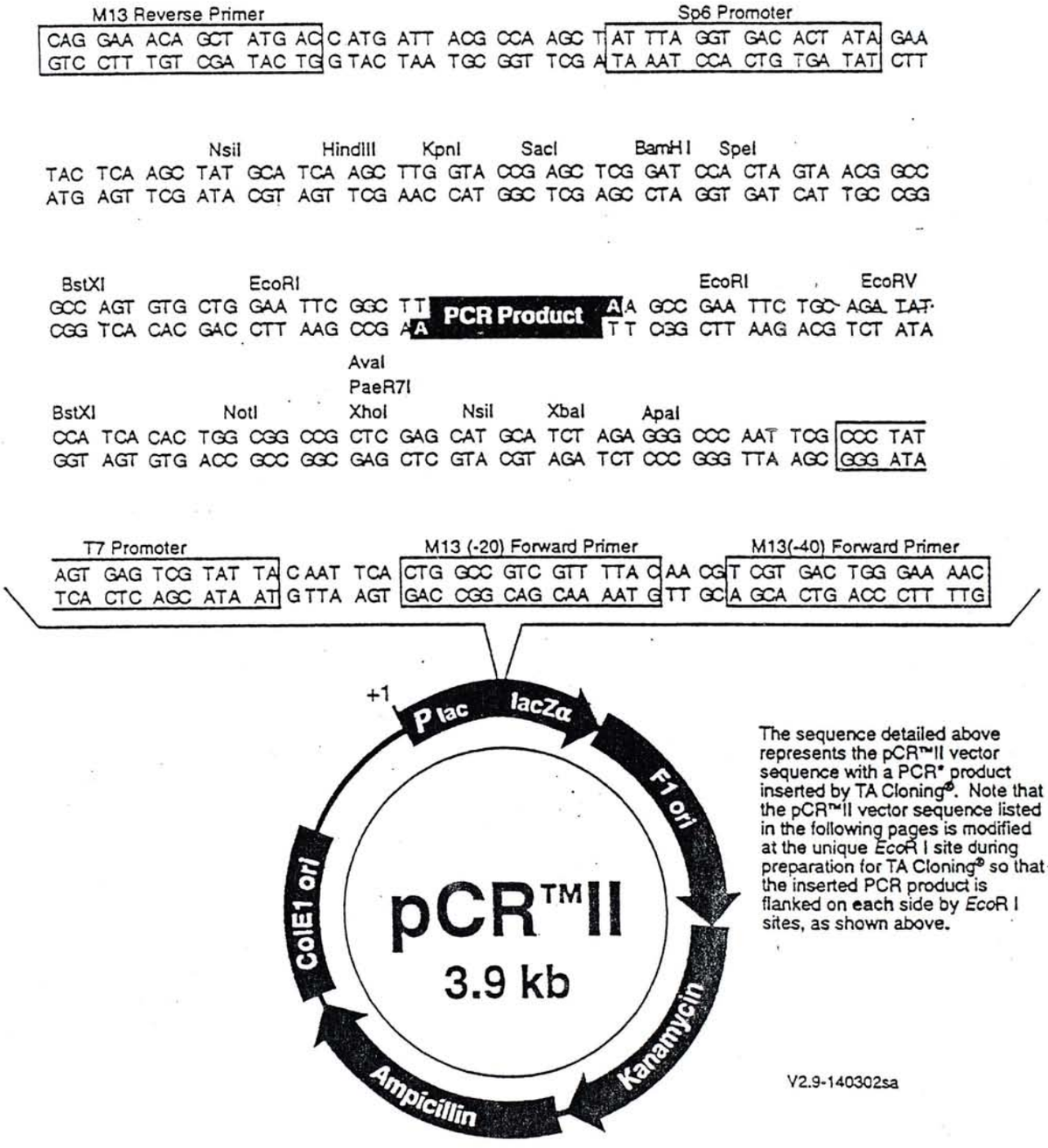

Fig.A1: pCRII +/- vector and the multiple cloning site sequence.

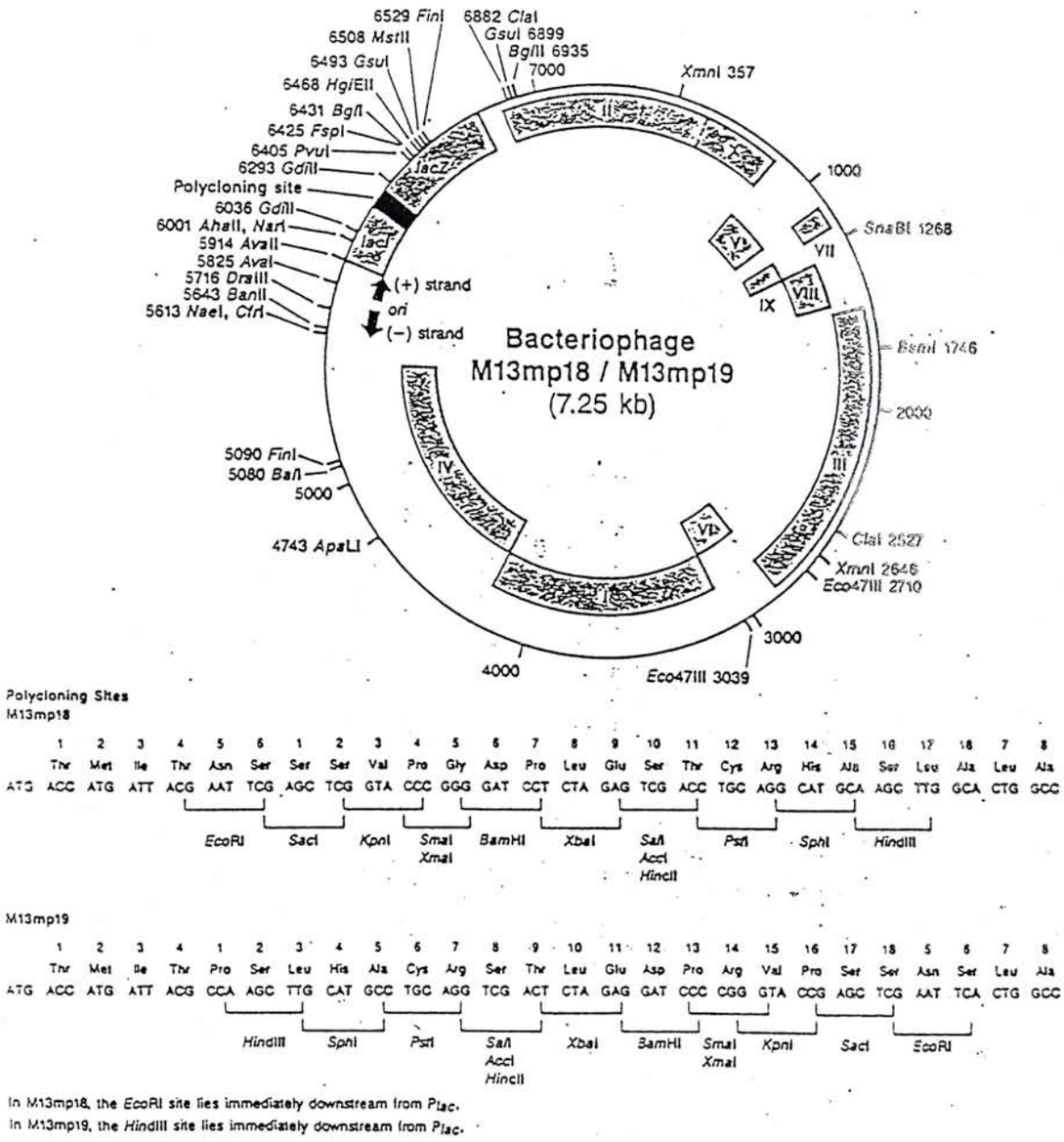

Fig.A2: Bacteriophage M13 vectors M13mp18 and M13mp19 and the multiple cloning site sequence.